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Obituary Notice

DOROTHY JORDAN LLOYD

Dr Dorothy Jordan Lloyd, Director of the British Leather Manufacturers' Research Association, died on 21 November 1946, at the age of 57. She was a biochemist in the truest sense. She was trained as a zoologist, and through the study of biological processes acquired a passionate interest in the chemistry of the most complex molecules that life produces.

She came of a medical family with academic associations, her father being Professor of Operative Surgery at Birmingham University. From King Edward School she came up to Newnham in 1908 and obtained a first in both parts of the Natural Sciences Tripos. Her research career began when she was awarded the Bathurst Studentship, and was continued as holder, from 1914 to 1920, of a Newnham College Fellowship. In 1916, at the early age of 27, she obtained her D.Sc. degree at London University. During the first World War she was associated with Professor [Sir F. Gowland] Hopkins and his staff in the Department of Biochemistry, Cambridge, on work of national importance under the Medical Research Council and remained with him until 1921. This period included a notable research with A. B. Clarke and E. D. McCrea on the subject of ropiness in bread.

Her appointment to the British Leather Manufacturers' Research Association (B.L.M.R.A.) in 1921 took her to the Lister Institute. From then onwards her life's work kept her in London, but her ties with Cambridge, and especially with Newnham and the School of Biochemistry, were always vigorously maintained.

One of the strongest influences in her scientific development was W. B. Hardy. There is, in fact, an interesting parallel in the course which each followed; both started as zoologists and began their research careers by studying physiological mechanisms in lower aquatic organisms, moving thence to the osmotic behaviour of the tissues and body fluids, and eventually reaching scientific maturity in the field of the physical chemistry of the proteins; both, in the sequel, devoted themselves to the application in industry of their scientific knowledge and experience.

Her work on gelatin and other proteins made Dorothy Jordan Lloyd a natural choice for the staff of the newly formed B.L.M.R.A. From that time onward, to her undiminished contributions to the fundamental physical chemistry of gelatin were added many others on the technical aspects of the manufacture and use of leather. In 1927, on Dr Pickard's resignation, she was appointed Director. The growth of the staff and laboratories under her leadership is a sufficient indication of her success in this office.

She did much more than satisfy the day-to-day technical requirements of the industry she served. Her efforts were equally directed to establishing a sound scientific background to the industrial processes of leather manufacture, and her efforts in this direction were recognized by the award, in 1939, of the Fraser Muir Moffat Medal of the Tanning Council of the U.S.A.

Perhaps no subject in biochemistry showed such rapid advances during the two decades of her directorship as that of the structure of the protein molecule, and in spite of her exacting professional life, never was she anywhere but in the front line of the attack on this problem. More than this, through her book, *The Chemistry of the Proteins*, first published in 1926 and later, in 1937, with Dr Agnes Shore in a revised edition, she put her own up-to-date and well-assimilated knowledge at the disposal of other workers in this field. There can be few happier examples of the gathering together within the same cover of accurate data and their useful application.

During the last year, her researches were showing increasing signs of fruition. Her last paper, on the swelling of protein fibres in organic solvents, which she herself was unfortunately unable to read, was contributed in September 1946 to the Faraday Society's Symposium on the swelling of proteins. In this paper one can see the threads of her earlier researches gathering together, and a picture clearly emerging of the structures and forces determining the form, and changes in form, of natural and artificial fibres. In this, as in all her work, her gift for applying advanced theory to the solution of practical technological problems can be discerned.

She took an active part in scientific organization, being from 1936 onwards a Member of Council, chairman of various committees, and eventually a Vice-President of the Royal Institute of Chemistry, a member of the Advisory Board of the Imperial Institute and from 1942 a Visitor of the Royal Institution.

If one seeks to express in a single word Dorothy Jordan Lloyd's predominant characteristic, that word is 'fearlessness'. It was in this spirit that she tackled the problems of an established industry—some of them so difficult to formulate as hardly to be thought susceptible to scientific attack. This characteristic also dominated her choice of relaxation. She was a notable mountaineer, and in later years a rider of steeplechasers. Her vigour in these pursuits, and in her equally adventurous intellectual excursions, will always be remembered by those who were privileged to enjoy them with her. She will be equally missed by her scientific colleagues, her friends in industry, and her partners in the pastimes she loved.

E. C. BATE-SMITH

Creatine and Creatinine Excretion in Women

By G. A. MAW, *Department of Biochemistry, University College, London*

(*Received* 31 *December* 1946)

Although references to a possible relation between urinary creatine and/or creatinine and the female sexual cycle have appeared in the literature during the last thirty years, the existence of such a relation still remains far from certain. The data of Krause & Cramer (1910, 1911) suggested that the onset of menstruation is associated with a creatinuria, but Rose (1917), Stearns & Lewis (1921), Hodgson & Lewis (1928), Hyde (1942) and others have been unable to confirm this. Smith (1942) has observed the appearance of creatinuria during menstrual flow and has claimed, in addition, to observe cyclic variations in the excretion of creatinine. In view of the connexion between sex and creatine metabolism, as exemplified by the occurrence of creatinuria with avitaminosis-E, the pronounced creatinuria of late pregnancy and puerperium, the effects produced by administered androgens on creatine excretion in children, etc., a recognizable correlation of this kind in the non-pregnant female would obviously be of interest. The following observations on physiological creatine and creatinine excretion have been carried out with a view to supplying more data on this possible relationship.

METHODS

Choice of subjects. Four female subjects provided daily 24 hr. urine samples for unbroken periods of 60–70 days. For comparative purposes, two male subjects were similarly studied, one for 23 days, the other for two periods of 16 and 40 days respectively. Details of the daily dietary constituents, including number of meat meals, excess water and carbohydrate above normal ingested, were recorded, together with an account of any exercise above normal taken. Single 24 hr. urines were provided by ten other male subjects, and ten female subjects provided samples over menstrual periods only. All subjects were within the age group 19–26 years and were resident members of a student hostel, living under closely similar conditions and subject to the same dietary variations. The diet was a normal one without supplementation, and of moderate protein content (total urinary N values ranged from 8·47 to 12·02 g./day).

Estimation of creatine and creatinine. Urine samples were preserved with toluene and analyzed immediately or kept at the longest 2 days stored at 4°. Creatinine was estimated by a modification of the usual procedure, with a Spekker absorptiometer with Ilford 604 filters. Creatine was converted to creatinine by the boiling method of Folin (1914), since the increased pigmentation of urine occurring in dehydration methods which involve autoclaving with picric or mineral acids gives high colour blanks, equivalent to up to 150 mg. creatine/l. This blank value, although it is a reproducible quantity and can be accurately allowed for, is undesirable since it is comparable with the actual amounts of creatine found to be present in some normal urines. Folin's original method gives a colour blank equivalent to less than 40 mg. creatine/l., and when correction for such a blank value is made, urinary creatine may be satisfactorily estimated down to concentrations of 20–30 ±5 mg./l.

RESULTS

Creatine excretion. In the urine of some 50 % of the female subjects studied, creatine was found to be occasionally present in amounts up to 150 mg./day, the daily excretion being generally in the region of 50 mg. No trace of creatine was found in the urine of any of the male subjects. The daily urinary creatine excretions of the four female subjects B–E are shown in Fig. 1. Only subject C (age 20, period of investigation 62 days) showed any extensive creatine excretion, the total weekly outputs being as follows: *0*, 123, 117, *161*, 66, 0, *77*, 276, 73 mg. The values in italics are those for weeks during which menstruation took place. Out of the eleven menstrual periods covered, two were associated with creatinuria, one with premenstrual creatinuria, one with a fairly continuous creatine excretion and the remaining seven with absence of creatinuria. The results for the second group of ten female subjects covering fourteen menstrual periods (seventy determinations) showed four associated with creati-

nuria and ten with its absence. In one of these subjects daily determinations made over a 45-day period showed a fluctuating creatinuria, ranging from 0 to 94 mg./day with an average level of *c.* 50 mg./day. No obvious or consistent relationship between creatine excretion, whenever it appeared in these two groups of subjects, and any phase of the menstrual cycle is apparent.

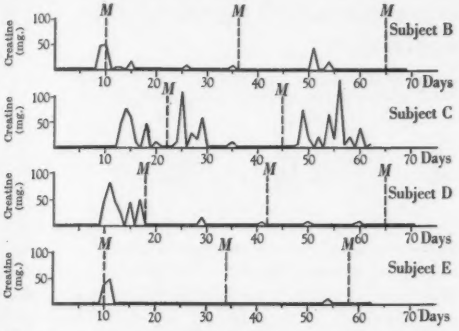

Fig. 1. Daily creatine excretion in subjects B–E. *M* signifies onset of menstruation.

Creatinine excretion. The daily creatinine excretion curves of the four female and two male subjects studied showed similarities both in the extent and frequency of the day to day fluctuations, and one typical graphical record only is therefore given (Fig. 2*a*), the data for the six subjects being summarized in Table 1.

Daily variations in creatinine output in men and women may be quite marked, and have been referred to by Cameron (1933), Espersen & Thomsen (1937), Dill & Horvath (1941), Wilkins, Fleischmann & Block (1941) and others, fluctuations of up to 25% of mean 24 hr. outputs being recorded. These observed variations undoubtedly represent the sum of a number of independent variations, each attributable to a separate source, and their consideration is necessary if a further cyclic variation of menstrual origin is to be established. Of the fluctuations which might exert a major effect on total excretion level the following have been considered: (*a*) a weekly periodicity resulting from a regular weekly environmental pattern, (*b*) irregular short term fluctuations caused by physiological compensatory mechanisms as a result of exercise, etc., and

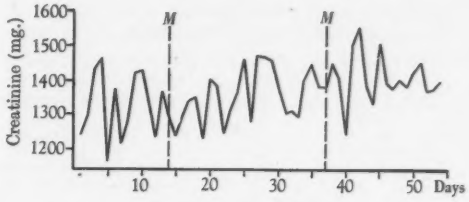

Fig. 2*a*. Daily creatinine excretion in subject C. *M* signifies onset of menstruation.

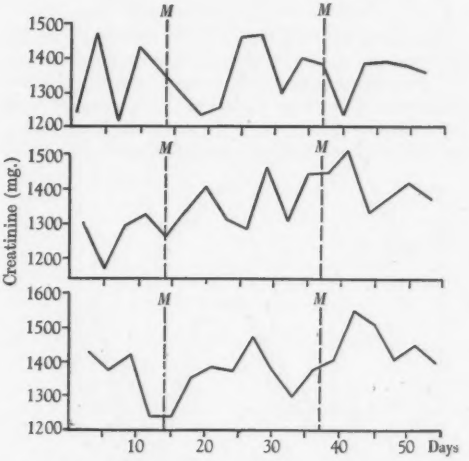

Fig. 2*b*. Creatinine excretion in subject C every third day. *M* signifies onset of menstruation.

(*c*) systematic trends due to growth, seasonal activities, etc. A periodic variation of type (*a*) might be expected in subjects on a hostel or hospital regimen with a fairly fixed weekly routine, and was looked for in the data of the four female subjects (the experimental periods of the two males not being

Table 1. *Creatinine excretion in male and female subjects*

Subject	Age	Period of study (days)	Average daily creatinine (mg.)	Maximum range of variation in excretion (mg.)	Maximum daily variation (mg.)
B, female	19	67	1306±102	421	391
C, female	20	62	1360± 90	382	298
D, female	26	70	1429± 84	330	233
E, female	19	62	1250± 80	394	253
A, male	19	40	1385±101	495	367
M, male	24	23	1866± 88	421	391

long enough to warrant detailed analysis of their data). The 24 hr. excretion values of each subject have been examined for variation between different days of the week and between weeks, by tabulation into columns of seven figures corresponding to the days of the week with omission of all values not covering a complete week. The results are given in Table 2.

Table 2. *Variation in creatinine excretion in four female subjects*

(Based on daily outputs in mg.)

Source of variation	Mean squares (degrees of freedom in brackets) B	C	D	E
Days	72633 (6)	21001 (6)	11084 (6)	11783 (6)
Weeks	105912 (6)	10335 (6)	17411 (6)	14432 (6)
Residual	342338 (36)	4613 (36)	4439 (43)	4448 (36)

The three subjects C–E exhibit mean squares for different days of the week significantly greater than the corresponding residual mean squares, which are measures of random variation. This suggests that some factor, dietary or environmental, is operating to produce a variation in excretion associated with the days of the week. In the case of subject B, however, the very large residual variation obscures any day of the week effect on the basis of an analysis of variance. The conclusion that the level of creatinine excretion is related to the days of the week is borne out by the curves in Fig. 3, which are plots of the mean excretion values for the different days of the week calculated as percentages of the average daily excretion over the whole experimental period, thus expressing the different sets of results in comparable terms. The marked rise and fall in all four curves points to a significant periodicity and further suggests that the same factor producing this effect is operable in subject B despite the greater random variations, since the curve for B clearly resembles those of the other three subjects.

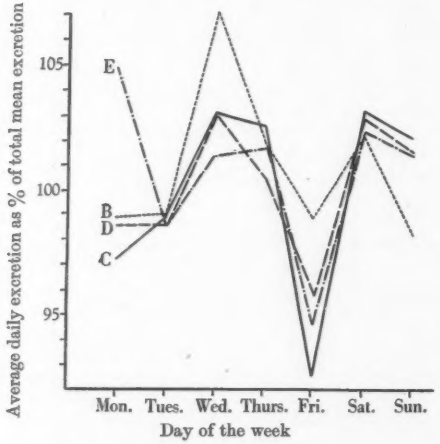

Fig. 3. Average daily excretion of creatinine in four female subjects.

Irregular short-term fluctuations with their probable compensatory characteristics are considered to be the major cause of deviations from the mean excretion level, but an interpretation of data minimizing or eliminating their effects might reveal the existence of more fundamental phenomena. Such a method of interpretation has been employed, although deductions from it are not necessarily significant owing to the difficulty of obtaining continuous data for a large number of subjects over many menstrual cycles.

The average creatinine outputs over the periods 1st–7th days, 2nd–8th days, 3rd–9th days, etc., have been computed, giving the mean daily excretion for weeks centred on days 4, 5, 6, etc., and these values have been plotted against the numbers of the middle days of the corresponding 7-day period. Each value is an average of one each of the days of the week so that the effects of both a weekly periodicity and sudden but compensatory variations occurring within the particular 7-day period will be largely eliminated. Further, each original daily excretion figure appears seven times in the moving averages so that any uncompensated variations will be spread out over periods of 3 days on either side of the day of their actual occurrence. This will effectively smooth out the large short-term fluctuations, but should not markedly affect a long-term periodicity such as a menstrual one. In addition, a plot of these 7-day moving averages should bring out steady trends in excretion level.

This treatment has been applied to the data for subjects B–E and male subject A and shows the females to give rise to two distinct types of excretion pattern. The curves for subjects C and D closely resemble one another, as do those of subjects B and E (Fig. 4). C and D exhibit a periodic rise and fall in daily output together with a pronounced tendency for the daily output to increase steadily with time. This general trend is represented by the sloping lines on the diagrams for these subjects. B and E show a less distinct periodicity, as far as can be judged

from the available data, and any trend in output is not so apparent. The general level of 7-day moving average excretion is given by the approximately horizontal broken lines on the diagrams. If we relate the curves to the occurrence of the menstrual phenomena, of the eight periods now falling within the period of investigation, five are associated with a fall in excretion level while three are associated with a rise. In six out of seven instances a maintained rise is apparent at the 14th day of the menstrual cycle.

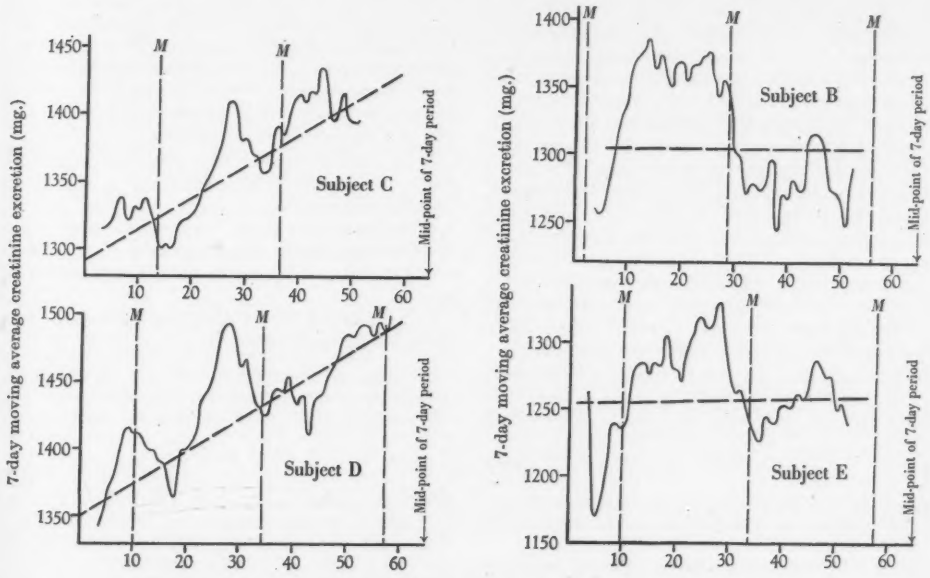

Fig. 4. Weekly moving average excretion of creatinine in four female subjects.

Comparable data for male subjects are not available, but the 40-day period of subject A shows a similar rise and fall in weekly average excretion over a period of about 28 days, and suggests that a long term periodicity may quite possibly be also a characteristic of males. On the basis of the data from these four subjects alone, there seem grounds for the existence of a menstrual effect on creatinine excretion. However, it is so strongly masked by other environmental effects that a substantial amount of further data would be required to establish it with certainty.

DISCUSSION

It is generally recognized that an intermittent physiological creatinuria is characteristic of some women and the data presented agree with this view. No obvious relationship between creatine excretion and menstruation has been observed in the fourteen subjects investigated. This is contrary to the observations of Smith (1942), who followed the output of urinary creatine and creatinine in four women on every second or third day for a number of cycles and found creatine appearing in the urine at the onset of menstruation, or rising to peak values in subjects normally exhibiting a creatinuria. It must be pointed out, however, that since all the other workers who have observed cases of significant creatine excretion in women have stressed its marked day to day irregularity, creatine excretion curves based solely on such periodic estimations cannot be justifiably interpreted as indicating any more than the qualitative existence of a creatinuria. There does not appear to be sufficient evidence from the data to hand, either in the literature or as a result of the present work, to support the existence of a clear-cut quantitative relationship.

As has been indicated, the daily excretion of creatinine in humans may vary by up to 300 mg. from day to day. Fluctuations of this order render meaningless the identification of cyclic changes in excretion from daily output curves alone. Day to day fluctuations in the six subjects in this study were frequently comparable with the maximum limits of variation over the whole period and masked any obvious cyclic changes. Nevertheless, analysis of the data for the females has revealed a significant correlation between level of creatinine excretion and

day of the week, presumably due to the influence of a weekly routine common to all subjects. It is probable that this weekly periodicity is a factor to be generally considered in all experiments on creatinine excretion in humans, and some such treatment of observations as that described must be used to eliminate this periodicity and also to reduce the effect of short-term fluctuations if particular deviations from the mean excretion level are being sought.

From excretion curves based on estimations made at intervals of 2–3 days, Smith (1942) has reported a regular premenstrual rise in excretion followed by a fall during the menstrual flow. Such curves smooth out the day to day fluctuations without taking into account their origin or their possible composition as a summation of several separately operating fluctuations, and thus give a false impression of the nature of creatinine excretion. To illustrate this, the data for the six subjects in this study have been interpreted in a similar manner, the representative results for subject C being given. In Fig. 2*b* are shown the three corresponding curves of values for every third day only, constructed from the complete excretion curve shown in Fig. 2*a*, and which resemble closely the curves obtained by Smith. It is obvious that not only are these 'periodic' curves unsatisfactory approximations of the complete excretion curve but that they bear no consistent relation to one another, and deductions drawn from them would have little significance. A connexion between menstruation and creatinine elimination has not been conclusively proved, and the data available indicate that if it is existent, it is by no means a clear-cut relationship as was proposed. The further long-term investigation of many subjects is clearly necessary, including examination of the excretion characteristics of males, before this relationship can be established with certainty. It is thought that treatment of results along the lines presented in this paper may be of value in clarifying this problem.

SUMMARY

1. Studies of the day to day creatine and creatinine excretion of a number of men and women, aged 19–26 years, revealed the absence of creatinuria in the males and its presence, in amounts up to 150 mg./day, in a proportion of the females.

2. No evidence has been obtained to relate creatinuria, whenever it appeared, to any phase of the menstrual cycle.

3. A weekly periodicity in creatinine excretion was observed, but there was no conclusive evidence to link the level of urinary creatinine with menstruation.

4. The nature of the daily fluctuations in creatinine output is discussed.

The author is indebted to Dr R. B. Fisher for his invaluable help in the analysis of the data obtained and to the University of London for a grant from the Dixon fund.

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Oxidation of Glucose, Glycerol and Acetate by *Staphylococcus aureus**

By DOROTHY M. POWELSON, P. W. WILSON and R. H. BURRIS
College of Agriculture, University of Wisconsin, Madison, Wisconsin, U.S.A.

(*Received* 2 *January*, 1947)

Because of the importance of *Staphylococcus aureus* as a test organism in assay of antibiotics, detailed information about its metabolism is desirable. While comparing the oxidation of glycerol and glucose in microrespiration experiments, we observed that resting suspensions rarely consumed enough oxygen for complete oxidation of these two substrates. Krebs (1937) also reported incomplete oxidation of glucose, glycerol, lactate and pyruvate by 18–24 hr. old cells of *Staph. aureus*. Usually about 2·5 mol. of oxygen/mol. of glucose is rapidly consumed followed by a slow uptake. The observed uptake of oxygen and respiratory quotients in our experiments could be accounted for if acetic acid is assumed to be an

* Supported in part by grants from the Wisconsin Alumni Research Foundation.

intermediate in the oxidation. This report summarizes results from quantitative respiration experiments that were undertaken to test this explanation and to determine if complete oxidation-reduction and carbon balances of the aerobic metabolism of *Staph. aureus* could be made.

METHODS

Preparation of cells. 100 ml. of the standard medium (10 g. Bacto-tryptone, 10 g. Bacto yeast-extract/l. distilled water, pH 7·2–7·4) in a 500 ml. Erlenmeyer flask was inoculated with 0·1 ml. of a 24 hr. broth culture of *Staph. aureus* NRRL-B313. The cultures were shaken mechanically at 30° until the desired density of cells was reached. After 6 hr. the cells were in the early phase of logarithmic growth and at 10 hr. in the middle of the logarithmic phase (see Fig. 3 for standard growth curve). When harvested, the cultures were cooled in an ice bath, then spun down in a Sharples or an angle-head centrifuge. The cells were washed twice with cold distilled water and resuspended in water to a density $(2 - \log G)$ of 0·96 (G is galvanometer reading) using a 720 mμ. filter and an 18 mm. tube in an Evelyn colorimeter. This optical density represents 3 mg. dry weight/ml. for cells in the period of logarithmic increase. The cell suspensions were used immediately. All distilled water was CO_2-free.

Oxygen and carbon dioxide. Warburg's method was used to estimate oxygen consumed and carbon dioxide liberated. To a 125 ml. Warburg flask were added 12·5 ml. of 0·1 M-phosphate buffer pH 7·0, 4 ml. distilled water, and 8 ml. cell suspension: 0·5 ml. of substrate solution was contained in a Keilin tube hooked by a platinum wire to the centre well. For determining O_2 uptake the well held 0·7 ml. of 10% KOH; for CO_2 liberated, KOH was omitted and 1·0 ml. of approximately 6 N-H_2SO_4 was placed in a side-arm.

Carbon. Total carbon at the start and end of the oxidation period was determined on the contents of the flasks used for estimation of CO_2. Immediately following the final manometric readings the flasks were removed to an ice bath. Analyses were made by a modification of the wet carbon method of Friedemann & Kendall (1929) on (*a*) samples of the flask contents, (*b*) cells centrifuged from the suspensions, and (*c*) the substrate added. The contents of the flasks that measured O_2 uptake were used to determine the remaining substrate, the acetate formed, and the carbon in the cells after respiration. Each flask was cooled at the end of a run, and the contents were transferred quantitatively to cold test tubes and centrifuged. The cells were removed and washed once with cold distilled water; the washing was added to the first supernatant fluid. Either a few drops of dilute acid were added to the cells to prevent slow respiration during storage in the refrigerator or the cells were kept frozen in the freezing compartment until analyses for carbon were made. The supernatant fluid was analyzed within 6–12 hr. Initial figures were obtained from analyses of controls kept in 125 ml. Erlenmeyer flasks that were treated in the same manner as the experimental material until the time of substrate addition. The controls were then cooled, the cells were removed, substrate was added to the supernatant fluid, and analyses were made.

Acetate. After removal of the cells, the supernatant fluid was adjusted with dilute NaOH and phenol red to pH 7·2 and concentrated *in vacuo* to about 10 ml. The concentrate was acidified to pH 2·5 with dilute H_2SO_4 and distilled under a residual pressure of 30–40 mm. Hg; the distilling flask was immersed in a water bath at 75–80°. Distilled water (50 ml.) was added from a delivery tube during distillation so that the volume of liquid in the distilling flask remained constant. The distillate was caught in 35 ml. dilute NaOH (190 μmol.); excess NaOH was titrated with standard 0·01 N-HCl. A blank with 10 ml. distilled water was run in the same manner. When standardized with known quantities of acetic acid, recovery with 50 μmol. of acetic acid ranged from 85 to 92%, but with 10 μmol. the variation was greater; 85% recovery was arbitrarily taken as the standard for the apparatus. The volatile acid in the titrated distillate was recovered by vacuum distillation into cold distilled water, made to 100 ml., and its identity checked by the Duclaux method. The distillate and the control acetic acid fractions were titrated with standard 0·01 N-NaOH.

Glycerol and glucose. Glycerol or glucose was determined on the residue in the distilling flask. Samples were analyzed for glycerol by the periodate method of Voris, Ellis & Maynard (1940). Using 0·002 M-KIO_4, 3% KI, and 0·005 N-$Na_2S_2O_3$, we could accurately determine 0·1–2·0 mg. of glycerol. In most trials glucose in the residue after distillation was determined by the copper-iodometric method of Somogyi (1937). In later experiments glucose was estimated by Folin & Malmros's (1929) micromethod, in a sample of the supernatant fluid before distillation.

RESULTS

In the first three experiments the oxidation of glycerol and glucose by *Staph. aureus* NRRL-B313 was studied. The results summarized in Figs. 1 and 2 and Tables 1 and 2 show that cells in the logarithmic period of growth (about 10 hr.) oxidized both glycerol and glucose to CO_2 and H_2O, but younger cells accumulated acetate. This finding suggested a test of the ability of cells of various ages to oxidize glycerol, glucose and acetate. As is evident in Fig. 3 and Exp. IV (Table 1), young cells of this strain hardly attack acetate, but later oxidize it (Fig. 4) at a rate comparable to those of glycerol and glucose. This physiological difference is accompanied by few other variations. The younger cells are about 1·5 times larger (in diameter) than the 10 hr. organisms, but both are encapsulated, Gram-positive and produce similar colonies.

It was thought that the physiological differences in the cells might be connected with a change in the medium used for their culture. Its initial pH of about 7·4 always decreased for 8–9 hr., then rose steadily to a final pH of 8·6–8·8, first reached at 24 hr. A change in colour of the cells from white to orange-yellow during this period did not appear to be an effect of the pH since cells grown for 12 hr. in a medium containing glycerol or glucose whose final pH was below 6·0 also showed this change in colour. The initial decrease in pH resulted from formation of a volatile acid by the growing cells; a Duclaux distillation identified the acid as acetic. Acetic acid

Table 1. *Respiratory balances for oxidations by* Staph. aureus *NRRL-B* 313

Experiment ...	I		II	III		IV		
	a	*b*		*a*	*b*	*a*	*b*	*c*
Substrate ...	Glycerol	Glucose	Glycerol	Glucose	Glucose	Acetate	Acetate	Acetate
μmol. substrate at start	93	50	88	51	49	102	48	98
μmol. substrate at end	17	1	7	2	1	97	5	64
μmol. O_2 respired	158	117	261	236	217	14	106	61
μmol. CO_2 formed	129	136	222	228	218	14	120	60
μmol. acetate formed	38	51	0	0	2	—	—	—
R.Q.	0·82	1·16	0·82	0·97	1·00	1·00	1·13	0·98
O/R*	1·06	1·16	1·00	0·97	1·00	1·00	1·13	0·98
Age of cells (hr.)	6	6	10	10	10	6	10	24
Growth density	0·126	0·126	0·611	0·620	0·620	0·111	0·638	0·959

* *Ratio* (*O*/*R*). The balance of oxidation and reduction during metabolism calculated as described by Johnson, Peterson & Fred (1931).

Table 2. *Carbon balances for oxidation of glycerol and glucose by* Staph. aureus *NRRL-B* 313

				Distribution of carbon					
		Total C in medium						Total	
Substrate	Time (min.)	(mg.)	Recovery (%)	Cells (mg.)	Substrate (mg.)	Acetate (mg.)	CO_2 (mg.)	(mg.)	Recovery (%)
				Experiment I. 6 hr. cells					
Endogenous	0	9·68	102*	9·01	—	0·07	0·01	9·09 94†	99‡
	180	9·85		8·73	—	0·12	0·17	9·02 92	
Glycerol	0	16·29	98	10·05	3·81	0·16	0·01	14·06 86	94
	290	14·37		9·90	0·60	1·08	1·55	13·13 83	
Glucose	0	15·54	102	10·44	3·66	0·18	0·01	14·29 92	93
	215	14·23		10·20	0·08	1·39	1·64	13·31 84	
				Experiment II. 10 hr. cells					
Endogenous	0	10·48	102	9·53	—	0·09	0·02	9·64 92	99
	380	10·10		8·91	—	0·08	0·57	9·56 89	
Glycerol	0	15·18	102	10·21	3·44	0·09	0·02	13·76 91	92
	380	13·43		10·00	0·34	0·09	2·10	12·53 81	
				Experiment III. 10 hr. cells					
Endogenous	0	11·40	99	9·78	—	0·00	0·02	9·80 86	99
	440	10·60		9·01	—	0·00	0·71	9·72 86	
Glucose	0	14·15	103	9·78	3·56	0·00	0·03	13·37 95	93
	440	11·82		9·52	0·16	0·00	2·73	12·41 85	
Glucose	0	15·15	99	9·54	3·66	0·01	0·02	13·23 88	93
	305	12·59		9·42	0·14	0·08	2·62	12·26 81	

* Recoveries in this column based on total carbon in medium + CO_2 at beginning and end. Total carbon in medium estimated by wet carbon method and would include cells, substrate, fermentation products other than CO_2, soluble products as lysed cells or polysaccharide.

† Figures in this column are (total C in analyzed products/total carbon in medium + CO_2) × 100.

‡ Recoveries in this column based on carbon found in products analyzed for at beginning and end.

equal to the quantity of volatile acid formed during the initial period decreased the pH of the sterile medium from 7·45 to 7·0.

Experiments were made to determine the effect of a higher initial hydrogen-ion concentration and the presence of acetate in the medium used for growth. The results (Exp. V, Table 3) show that production of the enzyme systems for oxidation of acetate was stimulated neither by a decrease in pH nor presence of acetate. Attempts to adapt 'resting' cells by allowing 6 hr. organisms to respire in the presence of acetate for 6–7 hr. were unsuccessful. As a possible source of a necessary coenzyme, boiled cells from a suspension which rapidly oxidized acetate were added but had no effect. Neither did growing the cells in a richer medium such as 10% liver extract-2% Bacto-tryptose broth.

The data of Exp. VI (Table 3) suggest that some change occurring in the medium during the growth of the organism is responsible for initiation of oxidation of acetate. Cells were grown in the standard medium until the growth density reached 0·620; such

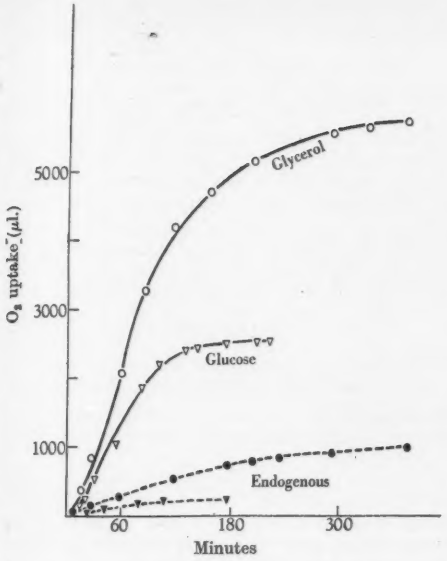

Fig. 1. Oxidation of glycerol and glucose by 6 hr. resting suspensions of *Staph. aureus* (Exp. I).

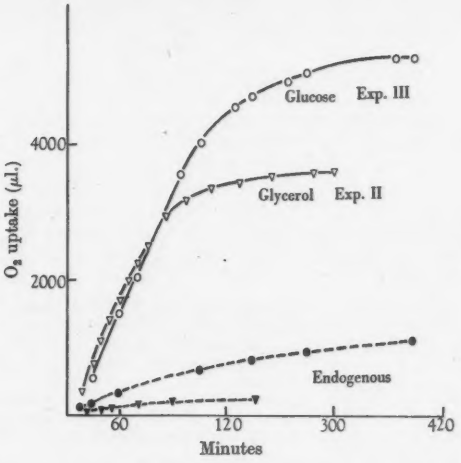

Fig. 2. Oxidation of glycerol (Exp. II) and glucose (Exp. III) by 10 hr. resting cells of *Staph. aureus*.

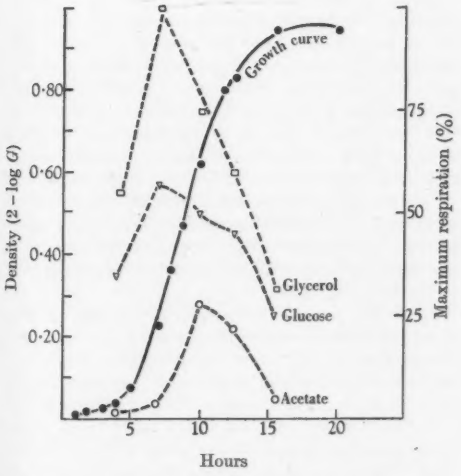

Fig. 3. Oxidation of glycerol, glucose and acetate by resting suspensions of *Staph. aureus* harvested at different phases of growth. •——• growth curve.

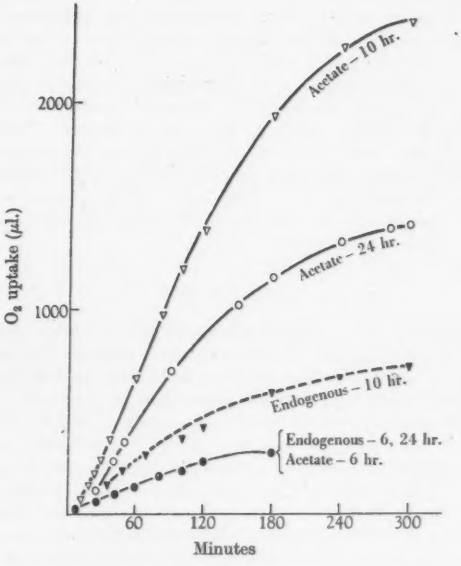

Fig. 4. Oxidation of 0·5 ml. 0·2 M-sodium acetate in 25 ml. total volume by 6, 10 and 24 hr. cells of *Staph. aureus*. Growth densities: 6 hr., 0·1024–0·1107; 10 hr., 0·629–0·643; 24 hr., 0·959.

Table 3. *Effect of changing growth medium on oxidation of acetate by young cells of* Staph. aureus *NRRL-B* 313

Medium	Incubation period (hr.)	Growth density	Q_{O_2} Endogenous	Q_{O_2} Acetate
		Experiment V		
1	6·5	0·1635	9·6	9·9
2	6·5	0·1177	6·4	5·7
3	6	0·1265	5·7	5·9
4	7	0·1357	5·6	6·5
		Experiment VI		
5	5·5	0·0605	6·7	6·8
6	9	0·0969	9·7	15·0
7	8·5	0·1369	6·5	16·8

Media used: (1) Standard, initial pH, 7·44, final, 6·90.
(2) Standard, initial pH, 7·08, final, 6·73.
(3) Standard plus 0·5% glycerol.
(4) Standard plus 0·5% acetate.
(5) Standard.
(6) Supernatant of 10 hr. culture, autoclaved, adjusted to pH 7·2.
(7) Supernatnat of 10 hr. culture, aseptically centrifuged. The cells from the 10 hr. culture (growth density 0·602) had an endogenous Q_{O_2} of 11·3; with acetate, 39·6.

cells are capable of oxidizing acetate. These were removed by centrifugation, and the supernatant fluid resterilized and inoculated as usual. The growth was slower than in the fresh medium; it required 9 hr. to reach a growth density (*c.* 0·10) that corresponds to a physiological age of about 6 hr. for cells seeded into fresh medium. If, instead of sterilizing the supernatant, the centrifugation was performed aseptically and the residual cells allowed to serve as inoculum, a growth density of about 0·10 was reached in 8·5 hr. Such physiologically young cells from either treatment, in contrast to those grown in the standard medium, did oxidize acetate. This peculiar behaviour toward acetate is not possessed by all strains of *Staph. aureus*; tests of three others, one recently isolated from a case of osteomyelitis, revealed that acetate was oxidized by neither very young nor older cells.

DISCUSSION

Consideration of the quantitative aspects of the data in Table 2 leads to the conclusion that the major products of oxidation of glycerol and glucose by *Staph. aureus* NRRL-B 313 are simply CO_2 and H_2O. It also appears probable that at least part of the oxidation goes by way of acetate. If other products are formed, they do not accumulate enough to be regarded as a significant part of the oxidative metabolism. If based on total carbon in the medium (plus CO_2 evolved) before and after incubation, recoveries of 98–103% are obtained, usually slightly more than 100%. If, however, the recovery is based on carbon in identified constituents of the medium—cells, glucose, glycerol, acetate and CO_2—it averages about 93% in the presence of substrate and 99% in its absence. As is evident by a comparison of the figures in the third and last columns of Table 2, this disagreement apparently originates from an incomplete analysis for the constituents in the medium. Even before respiration begins (0 min.), the sum of the carbon in the individual constituents for which analyses were made accounts for only 90–95% of the total carbon found in the medium, that is, in the suspension of cells plus substrate. After the oxidation, the discrepancy is about the same in the absence of substrate but increases in its presence.

Since incomplete recovery occurs even in the absence of substrate or oxidation, and since most of the carbon present is in the cells, it appears likely that the part unaccounted for arises from autolyzed cells and those not removed by centrifugation or from soluble cellular polysaccharide. Support for this view was secured in two experiments in which about 1·5 mg. of carbon was found in the supernatant fluid from 8 ml. of the cell suspension. This strain of *Staph. aureus*, as did many others tested, produced considerable quantities of gum in early growth, and capsules were easily demonstrated by the usual methods; chilled, concentrated suspensions formed a slimy mass.

Attempts to demonstrate polysaccharide formation sufficient to account for the missing carbon met, however, with only limited success. The supernatant fluid from cells after endogenous respiration and after oxidation of glucose was hydrolyzed with N-H_2SO_4 for 30 min. at 121°; only 10% of the missing carbon appeared as glucose after the hydrolysis. This result might not be conclusive, however, since Fellows & Routh (1944) found that young cultures of two pathogenic strains of *Staph. aureus* produce antigenic polysaccharides that are difficult

to hydrolyze. Our attempts to block synthesis of polysaccharide with dinitrophenol were unsuccessful.

Kendall, Friedemann & Ishikawa (1930) have also reported incomplete recovery of carbon from alanine, glucose and pyruvate when resting cells of *Staph. aureus* supplied with these substrates were aerated with CO_2-free air in tall cylinders. They report as both aerobic and anaerobic end products: lactate, volatile acid, CO_2 and small quantities of pyruvate. Test for lactate by the method of Barker & Summerson (1941) revealed less than 0·4 μmol. after oxidation of 50 μmol. of glucose by 10 hr. cells of *Staph. aureus* NRRL-B313.

SUMMARY

1. In microrespiration experiments resting cells of *Staphylococcus aureus* from the logarithmic period of growth (10 hr. cells) oxidized glycerol and glucose almost completely to CO_2 and H_2O. Younger organisms (6 hr. cells) oxidized these substrates to acetate, CO_2 and H_2O.

2. In these products 10% of the glycerol and 15% of the glucose did not appear. Lactate was not formed in appreciable quantity, but synthesis of polysaccharide might account for the missing source of carbon. Encapsulation of young cells favours this explanation, but direct quantitative evidence in its support was not obtained.

3. Study of this strain of *Staph. aureus* at different stages of growth established that only after the organism was well in the logarithmic phase did resting suspensions oxidize acetate. The effect was not one of simple adaptation but was associated with some as yet unknown change in the growth medium.

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Stability of Haemoglobin and of Certain Endoerythrocytic Enzymes *in vitro*

By D. KEILIN and Y. L. WANG, *Molteno Institute, University of Cambridge*

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In the present paper we propose to deal with those aspects of the stability of haemoglobin and of certain enzymes which can be expressed in terms of time during which these proteins may retain their specific biological activities and therefore their structural integrity. The study of this aspect of protein stability, which can be described as the life span of a protein, can hardly be based upon experiments solely designed for this purpose since it must depend to a very great extent upon material collected a considerable time ago and for different purposes.

MATERIAL

The material used in the present investigation was composed of samples of whole blood obtained from different sources:

(A) Samples of citrated horse blood collected by Dr E. G. D. Murray in 1922.

(B) Samples of horse blood received by the late Prof. G. H. F. Nuttall in 1904.

(C) Samples of guinea-pig blood collected by Nuttall in 1902.

(D) Samples of blood of other animals (jackal, ant bear, sea lion and Himalayan bear) received by Nuttall in 1901–3.

These samples of blood were investigated in respect of the properties of (1) haemoglobin, (2) carbonic anhydrase, (3) catalase, (4) glyoxalase and (5) choline esterase. Not every specimen was examined under all these headings, but in every case (except blood samples D) when the properties of haemoglobin or the activities of an enzyme were determined they were invariably compared with those of a corresponding sample of fresh blood.

METHODS

Haemoglobin and its derivatives

The methods applied to this study can be summarized as follows:

The haemin content of the blood was estimated as pyridine haemochromogen using as standard a pyridine haemochromogen prepared from pure haemin crystals. The determinations were carried out by means of (*a*) a microspectroscope and double wedge trough, (*b*) a Hilger-Nutting spectrophotometer, or (*c*) a King photoelectric colorimeter (1946).

For this purpose a stock solution of alkaline haematin containing 1 mg. haemin/ml. was prepared from pure haemin crystals dissolved in 0·1 N-NaOH. Of this stock solution 1 ml. was mixed with 4 ml. pyridine, brought to 25 ml. with distilled water and reduced with a few mg. of $Na_2S_2O_4$ to give a standard solution of pyridine haemochromogen. Samples of horse blood were converted to haemochromogen as follows: 2 ml. of 1 in 100 solution of blood were mixed with 0·4 ml. N-NaOH and 0·8 ml. pyridine, the mixture being diluted to 5 ml. with water and reduced with a few mg. $Na_2S_2O_4$. The pyridine haemochromogen thus obtained was compared with the standard solution by the above methods.

The absorption spectra of different samples of haemoglobin were determined in the usual way with the Hilger-Nutting spectrophotometer, using for this purpose blood diluted 1 in 100. As the haemoglobin in old samples of blood has a tendency to oxidize gradually to methaemoglobin, spectrophotometric studies were also carried out on samples treated with $Na_2S_2O_4$ and CO.

The oxygen capacity of blood samples was determined in Barcroft differential manometers. The right-hand flasks of the manometers receive 1 ml. of blood mixed with 2 ml. of distilled water and, in the case of fresh blood, a few mg. of saponin are added to assure the complete lysis of the red blood corpuscles. A volume of 0·3 ml. of a saturated solution of $K_3Fe(CN)_6$ is delivered from the dangling tube after the equilibration of the temperature and closing of the manometer taps. The left-hand flask in these experiments contains 3·3 ml. water.

The oxygen dissociation curves of haemoglobin were determined spectroscopically by a method previously described (Keilin & Wang, 1946). It consists in matching the absorption spectra of a solution of haemoglobin equilibrated at different pressures of O_2 in special microtonometer tubes with the absorption spectra of optical mixtures of oxyhaemoglobin and reduced haemoglobin kept separately in the two compartments of a double wedge trough. For this purpose, 1 ml. of blood is mixed with 3 ml. water and when lysis is complete (in the case of fresh blood) 1 ml. 0·25 M-phosphate buffer of pH 7·3 is added to the mixture. The stromatin is centrifuged off to give a clear solution in which the haemoglobin concentration is one fifth of that of the original blood. The spectroscopic measurements were carried out by means of a microspectroscope outfit and a double wedge trough placed close to the side aperture of the spectroscope on a brass platform attached to the microscope. The microtonometers used in these experiments were of the bulb-capillary type devised by Fox (1945) for the study of the oxygen affinities of invertebrate haemoglobins. The diameters of the capillary portions of these tonometers were carefully determined and one drop of blood was introduced into each bulb. Gas mixtures of the desired O_2 pressure were prepared by diluting air with oxygen-free nitrogen in a graduated mercury gasometer. The gas mixtures were passed through a series of bubble counters containing blood of the same concentration and treated with $K_3Fe(CN)_6$ in order to remove the O_2 bound to haemoglobin. About 400 ml. gas was then passed during 10 min. through the tonometer, which was occasionally rotated. Five tonometers filled with different gas mixtures were rotated for 15 min. in a water-bath at 21°. The blood was then drawn into the capillary and matched spectroscopically against the optical mixture of oxyhaemoglobin and deoxygenated haemoglobin in the double wedge trough (see Keilin & Wang, 1946).

Enzymes

The estimations of the activities of carbonic anhydrase, catalase, glyoxalase and choline esterase in fresh and old samples of blood were carried out manometrically. The brief descriptions of the methods used which follow are based upon the study of fresh samples of blood. The slight modifications of these methods introduced for certain samples of blood will be given later on.

Carbonic anhydrase. The activity of carbonic anhydrase was determined with the aid of the boat apparatus as described by Meldrum & Roughton (1933). One side of the glass boat receives 2 ml. of phosphate solution prepared by mixing equal volumes of 0·2 M-Na_2HPO_4 and 0·2 M-KH_2PO_4 while in the other side are placed 2 ml. of 0·2 M-$NaHCO_3$ in 0·02 M-NaOH. In the non-catalyzed reaction, which is used as a blank, the phosphate side of the boat receives 0·2 ml. water; in the catalyzed reaction it receives 0·2 ml. of blood diluted 1 : 200. The experiments were carried out at 0° with the boats shaken longitudinally 300 times/min.; the readings being taken every 5 sec. for about 1 min.

Catalase. The activity of catalase was determined both with differential manometers at room temperature and with the boat method at 0°. In the first case the right-hand flasks of the manometers received 0·3 ml. of 0·5% (v/v) blood, 2·7 ml. 0·1 M-phosphate buffer pH 6·5, while 0·3 ml. of 1 vol. H_2O_2 was put in a dangling tube. The left-hand flasks received 3·3 ml. water. The dangling tube was dislodged after equilibration of the temperature and the readings were taken every min. for 5 min., the manometers being shaken rapidly (about 200 strokes/min.).

In experiments with the boat method one side of the boat received 1·8 ml. water and 0·2 ml. of 1% (v/v) blood whereas the other side received 1 ml. of 2 vol. H_2O_2 mixed with 1 ml. 0·25 M-phosphate buffer pH 6·8. The activities were calculated from the evolution of O_2 during the first 3 min. of the reaction, the readings being taken every 10 sec. during the first minute and every 30 sec. during the last 2 min.

Glyoxalase. The activity of this enzyme was estimated in differential manometers by the rate of evolution of CO_2 from a bicarbonate buffer by lactic acid formed from methyl glyoxal in the presence of glutathione. In these experiments, carried out at room temperature, the right-hand flask of the manometer receives 0·2 ml. blood, 0·5 ml. of 0·18 M-$NaHCO_3$, 2·3 ml. water containing a few mg. glutathione, and 0·2 ml. of 5% (v/v) methyl glyoxal was put in a dangling tube. The left-hand flask receives the same

reagents, except the blood, which is replaced by 0·2 ml. water. The manometers are filled with the gas mixture composed of 95% N_2 and 5% CO_2. After the temperature equilibration the taps of the manometers are closed, the dangling tubes are dislodged and the readings are taken during 40 min.

Choline esterase. The activity of this enzyme was estimated by the liberation of CO_2 from bicarbonate by the acetic acid formed during the catalytic hydrolysis of acetylcholine. In this case the method adopted was similar to that used for the estimation of glyoxalase activity. The right-hand flask of the manometer receives 0·2 ml. blood, 0·5 ml. of 0·18 M-$NaHCO_3$, 2·3 ml. water and the dangling tube contains 0·3 ml. of 0·087 M-acetylcholine. The manometers are filled with N_2 containing 5% CO_2.

RESULTS

Horse blood A preserved 24 years

These blood samples were in several sealed ampoules of 2·5 ml. capacity, each containing about 2 ml. citrated horse blood collected aseptically in October 1922 by Dr E. G. D. Murray* then working in the Field Laboratories near Cambridge. The blood was specially collected for one of us (D.K.) in connexion with certain physiological experiments on the larvae of *Gastrophilus* parasitic in horses.

The ampoules were kept in cotton wool and enclosed in tin boxes deposited in a cold room at +1° in the Low Temperature Research Station, Cambridge. At the end of the year only one box containing eight ampoules remained from previous experiments. In June 1923 the box was transferred to the Molteno Institute where it remained exposed to the usual variations of room temperature. This box, although occasionally moved, was seldom opened so that the ampoules were well protected from light.

Haemoglobin in blood A

The haemoglobin of these samples was examined for the first time by one of us (D.K.) in November 1930, i.e. when the samples were about 8 years old. It was then noted that the blood was laked and of a purple colour showing the characteristic absorption band of deoxygenated haemoglobin when examined spectroscopically through the narrow portion of the ampoule. On opening an ampoule and transferring its contents to a test tube the haemoglobin was rapidly oxygenated to oxyhaemoglobin showing a normal colour and absorption spectrum. The oxygen capacity of this blood sample measured in the usual way was found to be approximately the same as an average capacity of three samples of freshly collected horse blood.

The blood in these ampoules was probably lysed and deoxygenated within the first few weeks of storage. The order of events as indicated by freshly stored samples is a gradual lysis beginning about 2 weeks after sealing the ampoule, accompanied by the deoxygenation of oxyhaemoglobin and its oxidation to methaemoglobin which is ultimately reduced back to haemoglobin. The oxygen is slowly used in oxidation processes within the cellular elements of the blood. The autoxidation of haemoglobin to methaemoglobin during the gradual disappearance of O_2 confirms some of the previous observations by Neill & Hastings (1925) and Brooks (1935) who found that the rate of oxidation of haemoglobin by molecular oxygen is greater at a small partial pressure of oxygen.

The ampoules were again examined between August 1945 and November 1946, i.e. after being stored for about 24 years. Before opening them it was ascertained that haemoglobin was in the deoxygenated state showing the characteristic colour and absorption spectrum. On opening an ampoule and transferring its contents to an open vessel, the haemoglobin became fully oxygenated without formation of methaemoglobin. The oxyhaemoglobin thus formed has the normal colour and absorption spectrum. It can easily be deoxygenated by diluting it with water and boiling it at room temperature in a Thunberg vacuum tube or by treating it with $Na_2S_2O_4$. In both cases it forms a typical haemoglobin showing one wide and somewhat diffuse band at about 550–560 mμ. The fact that there is no marked reinforcement at 557 mμ. denotes the absence of a haemochromogen which would appear, especially in presence of $Na_2S_2O_4$, had the haemoglobin undergone a partial denaturation. One may naturally ask whether the denatured fraction of haemoglobin, if formed, has undergone a further degradation beyond the stage at which it may still give a haemochromogen. However, this was ruled out by the fact that the total iron of these samples estimated with the $\alpha\alpha'$-dipyridyl method as previously described (Keilin & Hartree, 1945) was found to be within 3% of the amount calculated from the haemin content of the blood.

Before we enter into a discussion of the haemin content and the oxygen capacity of blood A as compared with those of fresh samples of horse blood it is important to mention that in both cases blood was drawn from the jugular veins of horses kept at the Field Laboratory and bled at more or less regular intervals. It is therefore not surprising that the fresh blood samples show a certain variation in their haemoglobin content. The haemin content and the oxygen capacity of blood A and of five different samples of fresh horse blood were determined as described above (p. 492). The results of these determinations, which are summarized in Table 1, clearly show that there is little difference in this respect between the 24-year-old blood A and the freshly

* Now Professor of Bacteriology, McGill University, Montreal.

collected material. Moreover, the O_2 binding capacity of blood/mg. of haemin which is 32·0 μl. for the old blood agrees well with the average of the values found for fresh blood (32·2) and is only about 6 % below the theoretical value of 34·2 μl.

Table 1. O_2 *capacity and haemin content of a 24-year-old sample of blood A and of fresh samples of horse blood*

(Blood samples 1, 2, 4, citrated; 3, 5, 6, defibrinated)

Samples of blood	O_2 capacity (μl. O_2/ml. blood)	Total haemin of blood (mg./ml.)	μl. O_2/mg. haemin
1. Old blood A	200	6·25	32·0
2. Fresh blood	190	6·40	29·7
3. Fresh blood	231	6·80	34·0
4. Fresh blood	202	6·50	31·5
5. Fresh blood	170	5·30	32·1
6. Fresh blood	176	5·09	34·7

The spectrophotometric curves of HbO_2 and HbCO in blood A and in fresh horse blood were almost indistinguishable and absolute values of the absorption in the region of the α- and β-bands of HbO_2 were found to be approximately the same (Table 2).

Table 2. *Heights of the α- and β-bands of oxyhaemoglobin in blood A and in two samples of fresh blood*

(Values expressed as the molecular extinction coefficient $\epsilon_{mol.} = 1/cl \log_{10} I_0/I$, where c = molar conc. of blood haemin, l = optical depth and I_0 and I are the intensities of incident and transmitted light.)

Samples of blood	$\epsilon_{mol.} \times 10^{-4}$ α—578 mμ.	β—542 mμ.
1. Old blood A	1·34	1·30
2. Fresh blood	1·38	1·35
3. Fresh blood	1·39	1·36

The oxygen dissociation curves of oxyhaemoglobin in the old sample A and in a fresh blood sample diluted five times were determined spectroscopically at 21° (cf. p. 492). These curves, represented in Fig. 1, show only small differences, the curve of the old sample being somewhat less S-shaped. The O_2 pressure (P_{50}), corresponding to 50 % HbO_2 at 21° is 5·1 mm. Hg for fresh and 4·8 mm. for

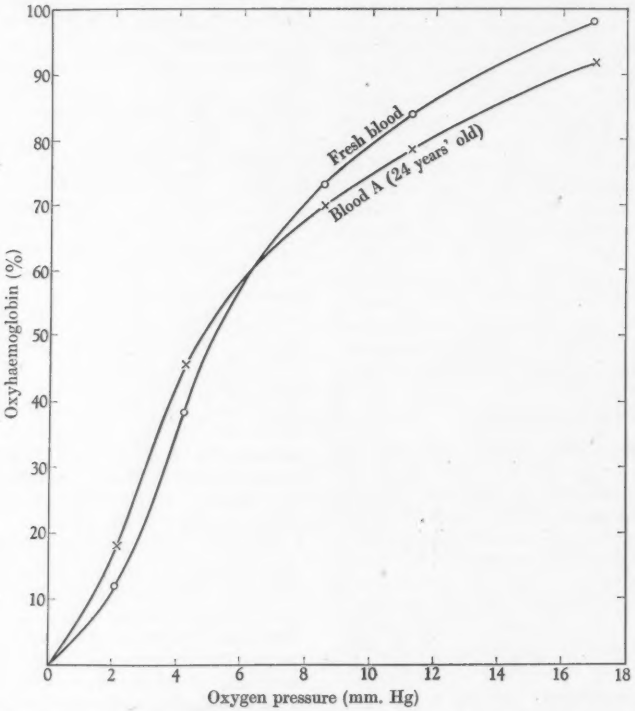

Fig. 1. O_2 dissociation curves of horse haemoglobin in a 24-year-old sample of blood A and in a fresh sample of blood at 21°.

the old blood A. Since an O_2 dissociation curve was obtained with only one sample of fresh blood, slight differences in the shape of curves shown in Fig. 1 may be partly due to individual variations.

Enzymes in blood A

Carbonic anhydrase. This enzyme, which catalyzes the reversible reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, is found in blood only within the red blood corpuscles. Its activity was determined by the boat method at 0° and expressed in mm. of manometric fluid corrected by the readings obtained in determinations of blanks or non-catalyzed reactions. Two experiments carried out with two different samples of blood A and with fresh blood gave the following results: In the first experiment the reaction catalyzed by fresh blood and by blood A gave readings of 205 and 165 mm. respectively, which shows that blood A retained 80 % of the normal activity. In the second experiment the corresponding values were 126 and 104 mm., indicating the retention of about 82 % of the normal activity.

Catalase. This enzyme, which catalyzes the decomposition of H_2O_2 to molecular O_2 and water ($2H_2O_2 = 2H_2O + O_2$), is also localized within the erythrocytes. Several samples of blood A were tested for the activity of this enzyme and compared with the catalase activity of fresh defibrinated, citrated or oxalated horse blood. The activities of one defibrinated sample of fresh horse blood and of one of the samples of blood A determined by the boat method at 0° and expressed in mm. of manometric fluid were 78 and 36 mm. respectively, hence the catalase activity in this sample of A was about 46 % that of the fresh blood. It is important to note that this ampoule was only two-thirds filled with blood. The catalase activity of a second sample of blood A, which almost completely filled the ampoule, as well as that of a fresh sample of oxalated blood were compared in differential manometers at 20°. In this case the evolution of O_2 in fresh blood and in the sample A during the first 2 min. of reaction were 83 and 72 μl. respectively, which shows that blood A has retained about 87 % of its catalase activity.

Glyoxalase, which is also an endoerythrocytic enzyme, catalyzes the conversion of methyl glyoxal into lactic acid ($CH_3COCHO + H_2O = CH_3CHOHCOOH$). The activities of this enzyme in fresh oxalated blood and in a sample of blood A were estimated manometrically in terms of CO_2 liberated from bicarbonate buffer in 10, 20 and 30 min. gave the following values: (*a*) for fresh blood 118, 257 and 372 μl. and (*b*) for samples A 58, 125 and 182 μl., which indicates that blood sample A retained about 50 % of its glyoxalase activity.

Choline esterase. In experiments on the estimation of choline esterase no distinction was made between the specific and non-specific esterases of corpuscles and plasma respectively. The activity of choline esterase was determined only by hydrolysis of acetylcholine and was expressed in μl. of CO_2 liberated from bicarbonate buffer. The volumes of gas evolved in 5, 10, 20 and 30 min. were as follows: (*a*) fresh oxalated blood: 36, 72, 120 and 170 μl. and (*b*) blood A: 15, 29, 57 and 75 μl. Blood A contains therefore about 42 % of its original choline esterase activity.

Blood samples in Nuttall's collection

This material was found among a large number of specimens of blood, sera and anti-sera collected and preserved by the late Prof. G. H. F. Nuttall between 1901 and 1904 and kept in boxes at room temperature. They were left over from his extensive serological investigations, the results of which were incorporated in the classical monograph on 'Blood immunity and blood relationships' published in 1904. In addition to blood specimens B and C, description of which will follow, Nuttall's collection contains a few other specimens of whole blood. Unfortunately some samples of whole blood which he received were not collected under aseptic conditions. They were sealed by him in a number of glass tubes with or without previous treatment with chloroform. We shall examine them separately as samples D. The anti-sera, obtained mainly from the blood of immunized rabbits and occasionally of guinea-pigs, as well as the sera of different animals were collected by him aseptically and preserved in sterile sealed tubes without addition of antiseptics. Many specimens were heated by him to 55°. Other samples of sera not aseptically collected had been passed through Chamberland filters before being sealed. Detailed descriptions of his methods for the collection and preservation of these interesting materials are given in his monographic study.

Horse blood B preserved 42 years

The blood B, although found in the above serological collection, was labelled in Nuttall's handwriting 'Horse sickness, received 13. vi. 1904, Gray'. It is possible that this sample was sent to him from South Africa in connexion with his work on blood parasites, especially piroplasmosis, in which he became interested soon after the completion of his serological study. This specimen of blood was found in two corked tubes each containing about 17 ml. leaving only 1–1·5 ml. air space. The corks were carefully sealed in with a thick layer of red sealing wax.

Haemoglobin in blood B

Examination of a thin film of blood near the air space showed that it was completely laked, giving a clear purple solution characteristic of a deoxygenated haemoglobin. On opening one of these tubes

and transferring about 2 ml. of the contents to another vessel the solution gradually turned red and showed distinct bands of oxyhaemoglobin, which on standing gradually oxidized to methaemoglobin, recognized by the appearance of an absorption band at 630 mμ.

A preliminary spectrophotometric study of this sample of blood revealed a marked deficiency in the haemoglobin content. Since this blood was obtained from a case of 'horse sickness' which, at that time, may have been confused with horse piroplasmosis or some other diseases, the low haemoglobin content of this blood sample could be ascribed to anaemia. However, the viscosity of this sample and the fact that it failed to dry when spread upon a slide suggested that the blood was diluted, probably with glycerol. To decide between these two possibilities 3 ml. of this blood were dialyzed against distilled water. Already after a few hours the water of dialysis contained much glycerol while the blood within the dialyzing sac rapidly increased in volume. After completion of dialysis the dry weight of the blood, corrected for the dilution, was found to be 75 mg./ml., whereas the value for a fresh sample of blood treated in a similar way was 230 mg./ml. This clearly indicates that the main reason for the low haemoglobin content of blood B was its dilution with glycerol which was added as a preservative.

The blood was then examined for the haemin content, oxygen capacity and absorption spectrum. Since it had a tendency to oxidize to methaemoglobin the spectrophotometric study of the sample was carried out after reduction with $Na_2S_2O_4$ and treatment with CO, followed by filtration through a dry kieselguhr bed. The results of these estimations (Table 3), confirm the previous conclusion, namely that the blood was diluted almost threefold.

Enzymes in blood B

Since the blood B was found to be diluted about three times, the quantities of this blood used for estimations of enzyme activities were proportionately increased to contain the same final concentrations of haemin as were present in the control experiments with fresh blood.

Carbonic anhydrase tested by the boat method and very rapid shaking at 0° gave the following results expressed in μl. CO_2 evolved between the 10th and the 40th seconds of the reaction: blank, 150 μl.; blood B, 605 μl.; and fresh blood, 690 μl. The relative catalytic activities of blood sample B and of fresh blood are therefore (605–150)/150 = 3·0 and (690–150)/150 = 3·6 respectively. The old blood thus retained about 83 % of its original carbonic anhydrase activity.

Catalase activity was determined both by the boat method at 0° and in differential manometers at 20°. In both series of experiments the rate of the reaction catalyzed by blood B was found to be from 75 to 92 % of the values obtained with different samples of fresh blood.

Glyoxalase activity was determined at 20° and expressed in terms of μl. CO_2 liberated from bicarbonate buffer by the lactic acid formed. In 10, 20 and 30 min., the CO_2 production was as follows: (*a*) fresh citrated blood: 174, 363 and 495 μl. and (*b*) an equivalent amount of blood B: 125, 246 and 340 μl. The glyoxalase activity of blood B is therefore about 70 % of that found in fresh blood.

Choline esterase activity was estimated at 20° in 10, 25 and 40 min. and gave the following values: (*a*) fresh oxalated blood: 60, 138 and 192 μl., and (*b*) an equivalent amount of blood B: 51, 120 and 180 μl. Blood B retained therefore about 85 % of the normal choline esterase activity.

Table 3. *Properties of haemoglobin in blood B (42-year-old sample) and fresh blood*

(Dry weight determined after dialysis; O_2 capacity determined manometrically; haemin as pyridine haemochromogen; $\epsilon_{mol.} = 1/cl \log_{10} I_0/I$.)

Blood	Dry weight (mg./ml.)	O_2 capacity (μl. O_2/ml.)	Total haemin (mg./ml.)	O_2 capacity (μl. O_2/mg. haemin)	HbCO $\epsilon_{mol.} \times 10^{-4}$ α – 570 mμ.	HbCO $\epsilon_{mol.} \times 10^{-4}$ β – 539 mμ.
Blood B 42 years old	75	70	2·30	30·3	1·34	1·35
Fresh blood	228	218	6·50	33·5	1·32	1·34

The fact that the value for the O_2 capacity of blood B determined manometrically is about 10 % below the value obtained by calculation from the haemin content shows that a small fraction of pigment was present in the form of methaemoglobin. This is more clearly shown by the normal values of the molecular extinction coefficient ($\epsilon_{mol.}$) of the HbCO, especially in the presence of $Na_2S_2O_4$.

Guinea-pig blood C preserved 44 years

Blood C comprised three 1·5 ml. ampoules each containing about 1 ml. of guinea-pig blood collected and preserved aseptically by Nuttall. They were placed by him, together with similar ampoules of sera containing different amounts of haemoglobin, into a box labelled 'Anti-reindeer. Guinea-pig, bled

17. iii. 1902.' Each of the three ampoules contained laked blood which was fluid, clear and of the purple colour characteristic of deoxygenated haemoglobin. This was confirmed by spectroscopic examination of the blood within the narrow parts of the ampoule.

It is well known that guinea-pig haemoglobin is one of the most easily crystallizable proteins. The ease of crystallization as well as the solubility depends, however, upon the state of the haemoglobin (Granick, 1942). Thus, oxyhaemoglobin of guinea-pig is very insoluble and easily crystallizes, forming characteristic tetrahedral crystals. Methaemoglobin is slightly more soluble and forms similar crystals, whereas haemoglobin is very soluble and does not crystallize under similar conditions. On deoxygenation crystals of HbO_2 rapidly dissolve and reform when the solution is reoxygenated. Crystallization of a protein is one of the properties which depend upon the integrity of its molecular structure. This property, however, could not be easily investigated in the old samples of horse blood A and B in which the haemoglobin was intimately mixed with the serum proteins. The old sample of guinea-pig blood, although laked and available only in very small quantities, offered an exceptional opportunity for the study of this property of haemoglobin in solutions preserved for more than 44 years.

On opening one of the ampoules of old guinea-pig blood (C) and transferring its contents to another vessel, the haemoglobin, thus exposed to air, slowly underwent oxygenation as shown by the change in its colour and its absorption spectrum. At the same time the solution became increasingly opalescent and viscous until it had practically solidified. Microscopic examination revealed a thick suspension of characteristic tetrahedral crystals varying greatly in size, the largest being almost visible to the naked eye. On reduction with $Na_2S_2O_4$ the crystals of HbO_2 were gradually deoxygenated and dissolved. The solution did not show any signs of denaturation.

Other specimens (D) of whole blood in Nuttall's collection

In addition to blood specimens B and C described above, Nuttall's serological collection contains a few other specimens of whole blood labelled as follows:

1. 'Common jackal (*Canis aureus*), received 27. xi. 1901, India.'

2. 'Ant bear, received 5. iii. 1903, removed to R.T. and sealed 9. iii. 1903.'

3. 'Sea lion (*Otaria californica*) received 24. vii. 1902, removed to R.T. and sealed 12. xi. 1902.'

4. 'Himalayan bear (*Ursus tibetanus* F. Cuv.) E. Asia, 27. xii. 1901 + chloroform, removed to R.T. and sealed 10. iii. 1903.'

These specimens of blood were collected apparently without aseptic precautions and after being kept in the cold they were transferred to room temperature (R.T.) and sealed in tubes. The blood of the Himalayan bear was the only specimen which was protected from putrefaction by treatment with chloroform.

(1) The 45-year-old samples of jackal blood were found to be in a fluid state and, before the tubes were opened, showed the colour and absorption spectrum of deoxygenated haemoglobin. Since there was no reinforcement in the region of the haemochromogen bands, the haemoglobin appeared to be solely in the native state.

On opening these tubes the blood was found to be saturated with gases and had a pungent odour of putrefaction. Exposed to air, the haemoglobin underwent oxygenation which was accompanied by slow oxidation to methaemoglobin. However, even after standing for several hours, the amount of methaemoglobin formed did not represent more than 10% of the total haemoglobin.

The oxygen capacity of this blood was estimated manometrically by the ferricyanide method as already described. As the blood was saturated with CO_2 it was found necessary to use rolls of filter paper impregnated with 10% KOH in the central tubes of the manometer flasks. Estimated in this way 1 ml. of blood was found to evolve 184 μl. O_2 which, corrected for about 10% methaemoglobin formed before the estimation, would give an oxygen capacity of 202 μl./ml. If we assume that the normal oxygen capacity of fresh jackal blood does not differ markedly from that of other mammals, the haemoglobin in the 45-year-old sample had not undergone any appreciable deterioration. This was corroborated by spectrophotometric determinations of the CO-haemoglobin in these samples.

(2) The 43-year-old blood of the 'ant bear' or 'aard-vark' (*Orycteropus capensis*) contained much solid material consisting of denatured proteins arising probably from a heavy bacterial infection present. The fluid fraction of the blood showed the colour and absorption spectrum of deoxygenated haemoglobin. The blood was saturated with gases and smelt very strongly of putrefaction. After filtration and boiling *in vacuo* in a Thunberg tube the clear solution of this blood was reoxygenated. During these manipulations about 10–12% of haemoglobin was oxidized to methaemoglobin. The oxygen capacity of this blood, estimated in the usual way and corrected for the methaemoglobin present, was 64 μl. O_2/ml. This blood sample therefore retained about 30% of its original oxygen capacity.

(3) The 44-year-old blood of the sea lion (*Otaria californica*), which also contained much solid material consisting of denatured proteins due to putrefaction, retained not more than 18% of its original oxygen capacity.

(4) The 45-year-old samples of bear's blood treated with chloroform were of a distinctly brown colour showing a diffuse spectrum of parahaematin which on reduction gave haemochromogen. The concentration of the latter was, however, too low to account for the complete disappearance of haemoglobin and for the loss of the oxygen binding property of the blood. This shows that the degradation of haemoglobin in this sample had proceeded beyond the stage of haemochromogen or even of the free haem.

Haemoglobin in laked blood is therefore less stable in presence of an organic solvent such as chloroform than in presence of micro-organisms producing putrefaction. The fact that the oxygen capacity of jackal blood was almost undiminished whereas those of the ant bear and of the sea lion were reduced to 30 and 18% respectively may be ascribed to differences in the bacterial flora responsible for the putrefaction. Attempts to study enzymes in these samples were abandoned since it appeared that they harbour viable micro-organisms.

Previous records of protein stability

The proteins which can be considered under this heading fall into three categories: (*a*) haemoglobin, (*b*) enzymes and (*c*) plant virus.

(*a*) *Haemoglobin.* Donaldson, Harding & Wright (1943), while discussing the permanence of the colour in standard tubes, write as follows: 'A tube which is the property of Dr Price-Jones and with which he carried out work on haematological standards (Price-Jones, Dill & Wright, 1931) was compared with the present standard. This tube was originally made by Dr Haldane more than thirty years ago, and had since been carefully preserved in the dark when not in use....' 'The agreement with the present standard is very close indeed. Although the exact colour of the tube when it was made is unknown, it is unlikely that it can have changed much when it still remains so close to other similarly prepared tubes.' However, since Haldane's standard tube contains CO-haemoglobin an appreciable denaturation of the pigment accompanied by the formation of CO-haemochromogen could hardly cause any marked change in the colour. Yet the results of our observations related above support the view that the haemoglobin in Haldane's standard tube remained unchanged from the time of its preparation.

(*b*) *Enzymes.* It was found by White (1909) that 'diastatic, fibrin-digesting and ereptic ferments' in seeds of wheat, maize, barley, oats and rye 'retain their activity without appreciable change in stored dry seeds for 20 or more years, that is long after the power of germination has been lost.'

More recently Steppuhn & Utkina-Ljubowzowa (1930) claimed to have demonstrated the presence of proteolytic enzymes in tissues of Egyptian mummies about 3000 years old and in those of a mammoth 30,000–100,000 years old. Unfortunately they give no quantitative data in support of these very interesting claims. Nor do they record any examination of the tissues for micro-organisms which might have invaded the tissues at various times and might have been responsible for the proteolytic activity.

(*c*) *Plant virus.* Thornberry, Valleau & Johnson (1938) have shown that 'Tobacco mosaic virus in cured tobacco that had been kept 52 years in a box, in a dry barn, was found to have retained some virulence.' Later Johnson & Valleau (1940) found that tobacco-mosaic-affected green leaves ground to a pulp and mixed with water retained about one-third of their original activity after 14 years, especially in presence of benzene or xylene.

DISCUSSION

The results of observations recorded in this paper show that the haemoglobin in old samples of blood (A and B) collected aseptically and kept for 24, 42 and 44 years retained its original properties such as oxygen binding capacity, the shape of the dissociation curve, spectroscopic properties and even the property of crystallization (blood C). Small quantitative differences between the old and fresh samples were well within the individual variations of fresh blood. There is therefore no indication that haemoglobin in the whole blood preserved for more than 44 years has undergone any appreciable change. In the same samples of old blood (A and B), enzymes such as carbonic anhydrase, catalase, glyoxalase and choline esterase retained up to 85% of their original catalytic activities.

All this shows that native proteins like haemoglobin and certain enzymes may retain their specific biological properties and therefore the integrity of their structure for more than 44 years in blood at room temperature.

The stability of haemoglobin and of endoerythrocytic enzymes *in vitro* contrasts markedly with their instability *in vivo*. In fact, since the life span of red blood corpuscles determined by different methods is approximately 120 days, our organism loses daily about 25 ml. of erythrocytes, which corresponds to about 8 g. of haemoglobin or 305 mg. of haemin. Yet neither free haemoglobin or its derivatives, nor any one of the endoerythrocytic enzymes such as carbonic anhydrase, catalase, glyoxalase or choline esterase are found free in the body. However, this rapid elimination of all components of the destroyed corpuscles is not due to the instability of these components but to active and well regulated processes of their degradation involving a series of catalyzed reactions localized in certain cells of the organism.

The analysis of conditions under which the above old samples (A, B and C) of blood were kept enables us to determine the factors which were either favourable or unfavourable for their preservation.

The four main factors which contributed to the stabilization of these blood samples were: (1) freedom from bacterial infection, (2) protection from light, (3) protection from oxygen and (4) high protein content.

(1) Freedom from bacterial infection of blood samples A and C was assured by their aseptic collection and preservation. In the case of the blood sample B it was partly due to dilution with glycerin.

(2) All samples were protected from light by being kept in closed tin boxes. On rare occasions and only for short periods were they exposed to light.

(3) The oxygen present in preserved samples of blood is derived partly from the small amount of air sealed within the ampoule or tube and partly from oxygen fixed by haemoglobin or dissolved in the fluid. Thus a 2·5 ml. ampoule containing 2·2 ml. blood A would contain 60 μl. O_2 in the air space, about 400 μl. O_2 combined with haemoglobin and 10–12 μl. dissolved in the fluid. The total amount of O_2 in different ampoules at the time of sealing probably varied between 450 and 500 μl. This O_2 was probably used up within a couple of weeks in normal respiratory processes of cellular constituents of the blood and very little of it remained for oxidative deterioration of native proteins or of their prosthetic groups. The same applies to blood specimens B and C, but not to blood samples D.

The factors unfavourable to preservation of these blood samples were: (1) bacterial infection in D, (2) the fluid state of the medium with its high water content which would facilitate any of the denaturation reactions likely to take place, (3) presence of hydrolytic enzymes liberated from lysed cellular elements, (4) a comparatively high temperature of storage and variations of this temperature, (5) presence of a small amount of O_2, at least during the initial period of storage, and (6) contact with glass.

It is important to note that although the 45-year-old samples of jackal blood showed marked signs of putrefaction the oxygen capacity was not far from that of fresh mammalian blood. On the other hand, in blood samples (Himalayan bear) protected from putrefaction by treatment with chloroform, haemoglobin was completely destroyed.

It is surprising that, although the conditions for the preservation of these blood samples were not ideal, the haemoglobin and the endoerythrocytic enzymes retained the major part of their specific biological activities and therefore the integrity of their complex molecular structure even after 44 years' storage. However, even under these comparatively unfavourable conditions we are still far from the upper limit of the life span of these proteins.

SUMMARY

1. Properties of haemoglobin and of certain endoerythrocytic enzymes were examined in 24- and 42-year-old samples of horse blood (A and B respectively) collected aseptically and preserved in the dark at room temperature.

2. Blood in these samples was laked and haemoglobin completely deoxygenated. The fact that no bands of haemochromogen could be detected even on addition of $Na_2S_2O_4$ indicates that no partial denaturation of haemoglobin occurred.

3. The O_2 capacity of these samples/mg. dry weight or/mg. haemin was approximately the same as that of fresh horse blood.

4. The O_2 dissociation curve of haemoglobin in sample A was found to be S-shaped and very little different from that of fresh blood, the P_{50} for blood A and for fresh horse blood being 5·1 and 4·8 mm. Hg respectively.

5. Absolute absorption spectra of oxyhaemoglobin in blood A and of carboxyhaemoglobin in blood B are indistinguishable from those of fresh horse blood.

6. From a 44-year-old sample of guinea-pig blood (C) collected and preserved aseptically, crystals of oxyhaemoglobin were obtained of the characteristic tetrahedral type similar in every respect to those prepared from fresh guinea-pig oxyhaemoglobin.

7. The endoerythrocytic enzymes such as carbonic anhydrase, catalase, glyoxalase and choline esterase in different samples of blood specimens A and B retained up to 85% of their activities when comparison was made with fresh horse blood.

8. The factors which favoured the preservation of haemoglobin and of the enzymes in these blood samples were mainly protection from bacterial infection, absence of oxygen, protection from light and a high concentration of proteins.

9. Since the conditions of preservation of these blood samples were not perfect, 44 years is probably very far below the possible life span of these proteins *in vitro*.

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Activity of the Cytochrome System in Heart Muscle Preparations

By D. KEILIN and E. F. HARTREE, *Molteno Institute, University of Cambridge*

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The best material for the study of the cytochrome system *in vitro* is the heart muscle preparation which we have previously described (Keilin & Hartree, 1938, 1940). The preparation consists of a uniform colloidal suspension of highly active material completely devoid of haemoglobin and suitable both for manometric and spectroscopic studies. In addition to the complete cytochrome system this preparation contains a powerful succinic dehydrogenase, α-glycerophosphoric dehydrogenase, fumarase and variable amounts of diaphorase, catalase and certain other enzymes, all bound to a protein the nature of which is not yet fully determined. This preparation is often referred to as cytochrome oxidase or as succinic dehydrogenase according to the nature of the substrate under investigation. The cytochrome oxidase activity of this preparation is determined by the rate of the catalyzed O_2 uptake in presence of an excess of cytochrome *c* and of a substance such as *p*-phenylenediamine, hydroquinone, adrenaline or ascorbic acid which, by reducing cytochrome *c*, ensure a constant supply of substrate for the oxidase. The activity of our preparation estimated in this way and expressed as Q_{O_2}, i.e. μl. O_2/mg. dry weight/hr. at 39° is about 1400 (Keilin & Hartree, 1940). Although this activity is well below that of several completely purified oxidizing enzymes all attempts to raise it have so far failed. More recently, by introducing a new method of preparation, Haas (1943) claimed to have extracted material containing 15 times more enzyme and being six times more active than the preparation obtained by our method. However, this claim is not supported by his own observations since the Q_{O_2} of his enzyme preparation was only 203 at 17° and was calculated by him to be 812 at 39° assuming that the activity of the preparation increases fourfold with this rise in temperature. The calculated activity of Haas's material is therefore not six times higher but 40 % lower than that of our preparation.

Since heart muscle preparations are often used for the study of cytochrome in relation to other intracellular catalytic systems we propose to compare the main properties of our preparation with that of Haas. We shall also examine the claims put forward by Haas (1943, 1944) (*a*) that the use of ultrasonic vibrations has led to the production of a soluble cytochrome oxidase and (*b*) that the oxidase may be separated into two protein components.

EXPERIMENTAL

Methods

O_2 uptakes. These were measured in Barcroft differential manometers at 39°. The oxidase sample was diluted to 3 ml. with 0·3 ml. cytochrome *c* (Keilin & Hartree, 1945) and phosphate buffer (0·1 M, pH 7·3). The substrate (*p*-phenylenediamine or hydroquinone) was added as 0·3 ml. aqueous solution from dangling tubes.

Enzyme units. In order to work out a balance sheet of the enzyme content at different stages of our preparation, samples of successive fractions were withdrawn, ground with sand and diluted with phosphate buffer (0·1 M, pH 7·3) to a known volume. The enzyme content was expressed in arbitrary units corresponding to the quantity of enzyme which catalyzes an O_2 uptake of 100 ml./hr. under the above standard conditions.

Cytochrome oxidase

This was prepared essentially as previously described. Finely minced horse heart muscle (500 g.) (*A*) was washed eight times with 5 l. tap water, the mince being squeezed out through muslin after each washing. The residual mince, 390 g. (*B*), was ground 1·5 hr. in a mechanical mortar with 100 g. sand and 500 ml. 0·02 M-phosphate buffer pH 7·3. The mixture was separated in a centrifuge into 690 ml. supernatant fluid (*C*) and a solid residue (above the layer of sand) which was resuspended in water to 690 ml. (*D*). The supernatant fluid was cooled by the addition of 100 g. crushed ice and the pH adjusted to 5·5 with N-acetic acid. The mixture was immediately centrifuged and the solid resuspended in 0·1 M-phosphate buffer pH 7·3 giving 247 ml. of the final preparation (*E*) containing the complete cytochrome system and a powerful succinic dehydrogenase. During the fractionation 20 g. of mince *A* was removed, ground with sand, diluted to 1 l. with 0·1 M-phosphate buffer pH 7·3, centrifuged to remove the sand, giving the fraction *A'*. 15·6 g. washed mince *B* treated in the same way gave fraction *B'*.

The activities of different fractions were estimated manometrically over a period of 30 min. as with some of the cruder preparations there was a slight lag at the beginning of the experiment which precluded the use of initial rates of oxygen uptake in estimating enzyme activity. Table 1, which gives the enzyme activities and enzyme contents of different fractions, shows that the final fraction of Q_{O_2} 1190 contains 42% of the activity of the unwashed mince. The Q_{O_2} of this fraction estimated from the initial O_2 uptake (5 min.) is about 1420 (Table 2). The apparent slight rise in enzyme content of minced muscle after washing may be due to the removal of some inhibitors.

Cytochrome oxidase according to Haas

Minced horse heart (500 g.) was used instead of the pig heart as originally specified. The method consists in grinding the unwashed mince with sand and toluene for 12 hr. and storing it overnight at 0°. Next day the mixture is ground with ammonia buffer pH 10·4 and centrifuged. The red fluid is then precipitated in the manner described above and resuspended in buffer pH 9·5 giving 742 ml. reddish opalescent fluid containing 11·4 mg. dry weight/ml. The activity of this preparation estimated with hydroquinone at 39°, as shown in Table 2, is higher than that calculated by Haas and is only slightly lower than that of our fraction *E* estimated under similar conditions. The yield by Haas' method is only 30% higher than by our method: thus the Haas preparation contains 109,000 units whereas our fraction *E* contains 72,500 units. However, this slightly higher yield has no practical advantage since no methods are so far available for further purification of this fraction and the quantity of material produced in one operation is always far in excess of that which can be used in the most extensive experiments. On the other hand the chief disadvantages of the Haas preparation are that it cannot be used for spectroscopic studies of cytochrome owing to the presence of denatured myoglobin and a partial destruction of component *b*, and that furthermore the preparation is useless for the study of succinic dehydrogenase which is rapidly destroyed at pH values above 9 (Keilin & Hartree, 1940).

Table 1. *Activities and yields of cytochrome oxidase in different fractions of heart muscle preparation*

(Samples tested in differential manometers at 39° with 0·3 ml. 1% cytochrome *c* and 20 mg. *p*-phenylenediamine; volumes made up to 3·3 ml. with 0·1 M-phosphate buffer pH 7·3. Dry weight determined after removal of buffer by dialysis.)

Source of enzyme	Dry weight (mg./ml.)	O_2 uptake (μl. O_2/30 min.)	Q_{O_2}	Total enzyme units/fraction
0·5 ml. *A'*	2·21	167	150	167,000
0·5 ml. *B'*	1·87	190	204	190,000
0·05 ml. *C*	14·1	333	940	94,000
0·05 ml. *D*	31·8	177	221	51,000
0·02 ml. *E*	23·0	275	1190	72,500

Table 2. *Activities of two heart muscle preparations, E (this paper) and Haas' oxidase*

(Activities tested manometrically at 39° with 0·3 ml. 1% cytochrome *c* and 18 mg. hydroquinone; volumes made up to 3·3 ml. with 0·1 M-phosphate buffer pH 7·3.)

Source of enzyme	O_2 uptake (μl. O_2/5 min.)	Q_{O_2}
0·025 ml. *E*	67	1420
0·05 ml. Haas' prep.	61	1280

Effect of ultrasonic vibrations

Haas has described the use of ultrasonic waves as a means of breaking down the aggregates which constitute oxidase preparations of the above type. This treatment reduces the proportion of the total solids which can be centrifuged down in a gravitational field of 10,000 *g* though the clear fluids so obtained show a marked Tyndall effect which demonstrates

that the particles are still much larger than the usual molecular size of purified proteins.

Moreover the experimental distinction between colloidal and true solution in these cases is purely arbitrary as the gradation of particle size is continuous. In true solution a protein must be present as a separate entity and not as a component of a large heterogeneous aggregate. A centrifugal field of 10,000 *g* which was used by Haas to remove the insoluble fraction of his preparation will leave in suspension all particles with a 'molecular weight' up to 10^8 which is considerably higher than the probable size of a single enzyme molecule. According to Haas the activity of his oxidase preparation was not raised by exposure to ultrasonic radiation: however, had the oxidase been brought into true solution it should have been possible to devise a suitable method for further purification. So far, no data have been brought forward to indicate that the irradiation was helpful in this respect.

Alleged separation of cytochrome oxidase into two components

Haas (1944) found that on ultracentrifuging his oxidase preparation, suitably diluted, a solid residue is obtained (component I) which on resuspension shows a much decreased activity in the oxidation of hydroquinone in presence of cytochrome *c*. By heating the undiluted oxidase preparation at 100° for 3 min. and centrifuging off the heat-denatured protein a thermostable yellowish solution containing component II, a protein, is obtained. The addition of the latter to the component I largely restores its catalytic activity, in other words two components are required for the oxidation of cytochrome *c* by molecular O_2. This suggests, according to Haas, 'that Warburg's oxygen-transferring enzyme and cytochrome oxidase are not identical but represent two individual enzymes'. We repeated Haas' experiment using *p*-phenylenediamine in place of hydroquinone and have confirmed his results (Table 3). However, the method of preparation of component II suggested to us the possibility of replacing it by some other native or denatured proteins. In fact, we found that component II can be successfully replaced by plasma proteins or by a very low concentration of haematin-free denatured globin obtained from recrystallized haemoglobin (Table 3). It is therefore impossible to regard the component II as an enzyme or as an essential cell catalyst. It affects only the colloidal structure of a preparation previously modified by ultracentrifuging and thus enhances the mutual accessibility of the components of the reaction.

Table 3. *Fractionation of Haas' cytochrome oxidase*

(Activities of fraction I and II of the oxidase compared with the activities of fraction I in presence of plasma or denatured globin. Samples tested manometrically at 39° with 0·3 ml. cytochrome *c* and 20 mg. *p*-phenylenediamine. Volumes made up to 3·3 ml. with 0·1 M-phosphate buffer pH 7·3. Fraction I equivalent to 0·1 ml. and fraction II to 1·0 ml. oxidase.)

Enzyme fractions	O_2 uptake (μl. O_2/25 min.)
0·1 ml. original oxidase	155
Fraction I	61
Fraction II	0
Fraction I + fraction II	129
Fraction I + 1% plasma proteins	136
Fraction I + 0·1% denatured globin	116

SUMMARY

1. The heart muscle preparation obtained by our method is a colloidal suspension of active material free from haemoglobin and containing a complete cytochrome system, succinic dehydrogenase and certain other enzymes.

2. The estimation of the cytochrome oxidase activity of this preparation in presence of an excess of cytochrome *c* and substances like *p*-phenylenediamine or hydroquinone at 39° gives a Q_{O_2} of about 1400.

3. Haas' preparation of cytochrome oxidase is less active and, owing to the presence of haemoglobin derivatives and to the high pH (> 9) reached during purification, cannot be used for the spectroscopic study of cytochrome or for the study of succinic dehydrogenase.

4. The treatment with ultrasonic waves as applied by Haas does not bring cytochrome oxidase into solution, or raise the activity of the preparation and so far has not assisted the further purification of the enzyme.

5. Haas' claim that cytochrome oxidase may be split into two component enzymes (I and II) is not justified since his enzyme II has been successfully replaced in our experiments by plasma proteins or by small amounts of denatured globin.

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Effect of Haematin on the Oxidation of Succinic Acid by Tissue Preparations

By D. KEILIN and E. F. HARTREE, *Molteno Institute, University of Cambridge*

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While investigating the possibility that free haematin might act as a carrier analogous to cytochrome *c* in the oxidation of succinate by heart muscle preparations, we found on the contrary that it had a marked inhibitory effect on the oxidation system. In order to analyze the mechanism of this inhibition we attempted to reverse it by addition of such substances as organic bases and denatured proteins which co-ordinate with haematin Fe.

MATERIALS AND METHODS

Heart-muscle preparation was obtained as described in the preceding paper (Keilin & Hartree, 1947).

Cytochrome c used in these experiments contained 0·34% Fe and was prepared as previously described (Keilin & Hartree, 1945).

Pigeon breast-muscle preparation. The breast muscles of 2 pigeons were passed through a Latapie mincer and stirred for 10 min. with 1 l. distilled water. The mixture was squeezed out through muslin and the cloudy fluid centrifuged hard. The resulting clear red fluid was rejected and the brown residue shaken gently with 100 ml. water to form a suspension of fine particles without disturbing the mat of fibrous material at the bottom of the tube. The suspension was diluted to 250 ml. with 0·2 M-Na_2HPO_4 and centrifuged for 5 min. The cloudy fluid, carefully separated from the solid material, was mixed with 100 g. crushed ice and the pH was adjusted to 5·5 by cautious addition with stirring of N-acetic acid. The precipitate was immediately centrifuged down and resuspended in 20 ml. 0·1 M-phosphate buffer pH 7·3.

Both the heart and the pigeon breast-muscle preparations can be preserved in a refrigerator for 4–5 days. Throughout this paper they will be referred to as 'oxidase preparations'.

Haematin solution. 2×10^{-3} M-crystalline haemin was made up in 0·1 N-NaOH and preserved in a well stoppered flask. When necessary, fresh dilutions were made with 0·1 N-NaOH.

Denatured globin. The procedure is based upon the method of Anson & Mirsky (1930) for the preparation of native globin. An amount of 30 ml. corpuscles, obtained from defibrinated blood and washed 3 times with 0·9% saline solution, was lysed with 60 ml. distilled water. 50 ml. 0·1 N-HCl was added and the mixture centrifuged in an angle centrifuge to remove stroma. The 100 ml. clear red fluid so obtained was run as a thin stream into a well-stirred mixture of 1 l. acetone and 20 ml. conc. HCl. The precipitate of denatured globin was filtered on a Buchner funnel and rapidly washed at first with acetone containing 1% conc. HCl and finally with pure acetone. The white powder was dried in air and preserved in a stoppered bottle. It is soluble in water but is completely precipitated on addition of alkali sufficient to bring the solution to pH 6–8.

The activity of the succinic system in the oxidase preparations was estimated aerobically in Barcroft differential manometers at 39°. The manometer flasks contained 0·1 ml. heart muscle or 0·04 ml. breast muscle preparation in 3·3 ml. fluid comprising 0·07 M-phosphate pH 7·3, 0·04 M-succinate and 4×10^{-5} M-cytochrome *c*. The addition of sodium succinate after temperature equilibration and closing of the manometer taps was effected by means of dangling tubes suspended by platinum hooks from the central tubes of the flasks.

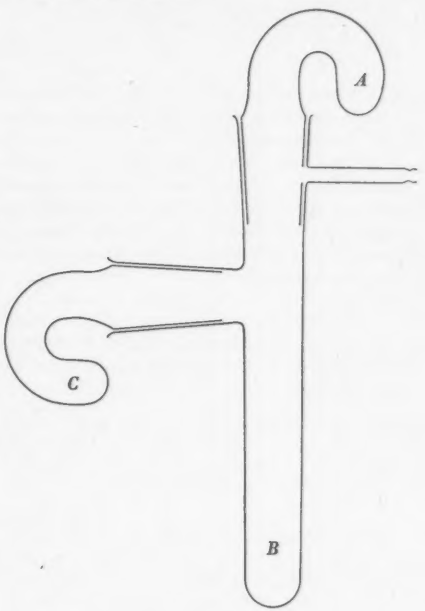

Fig. 1. Modified Thunberg tube (*B*) with two hollow stoppers (*A* and *C*).

Estimations of dehydrogenase activity by the rate of reduction of methylene blue were carried out either in Thunberg tubes with hollow stoppers in the usual way or in a special type of vacuum tube with an extra hollow stopper (Fig. 1).

RESULTS

The effect of haematin on the aerobic oxidation of succinic acid

Our experiments with the heart muscle preparation are summarized in Table 1. They show that 2×10^{-4}M-haematin strongly inhibits the oxidation of succinic acid by the oxidase preparation + cytochrome *c*. However, denatured globin and glyoxalin, provided that they are added before the haematin, protect the oxidizing system from this inhibitory effect. Whereas glyoxalin acts only as a protective substance, denatured globin does more than prevent the inhibition; it brings about a considerable acceleration of the reaction.* The addition of other denatured proteins or even of boiled oxidase preparation also protects succinic dehydrogenase from inhibition by haematin.

Table 1. *The oxidation of succinic acid by heart muscle preparation (oxid.); its inhibition by haematin and protection against this inhibition by glyoxalin and denatured globin*

(Estimations carried out manometrically at 39°: 0·1 ml. oxid.; 0·3 ml. 1% cytochrome *c*; 0·04M-sodium succinate; 2×10^{-4}M-haematin; 0·3 ml. 0·1M-neutralized glyoxalin; 0·3 ml. 1·4% denatured globin. Volumes made up to 3·3 ml. with 0·1M-phosphate buffer pH 7·3.)

Content of manometric flasks	O_2 uptake in 1st 15 min. (μl.)	Added at 15 min.	O_2 uptake in 2nd 15 min. (μl.)
Oxid. + cyt. *c* + succin.	110	—	—
Oxid. + cyt. *c* + succin. + glyoxalin	110	—	—
Oxid. + cyt. *c* + succin. + glyoxalin* + haematin	125	—	—
Oxid. + cyt. *c* + succin. + globin	233	—	—
Oxid. + cyt. *c* + succin. + globin* + haematin	215	—	—
Oxid. + cyt. *c* + succin. + haematin	4	Glyoxalin	24
Oxid. + cyt. *c* + succin. + haematin	4	Globin	20

*Added before haematin to oxid. + cyt. *c*.

The inhibitory effects of two concentrations of haematin (10^{-4}M and 4×10^{-5}M) tested under standard conditions with a wide range of succinate concentrations were found to be approximately constant. This shows that there is no appreciable competition between haematin and succinate; in other words, they do not compete for the same point of attachment on the succinic dehydrogenase protein.

Table 2. *The oxidation of succinic acid by a pigeon breast-muscle preparation (oxid.); its inhibition by haematin and protection against this inhibition by denatured globin*

(Estimations carried out manometrically at 39°: 0·04 ml. muscle extract; 0·3 ml. 1% cytochrome *c*; 0·04M-sodium succinate; 2×10^{-4}M-haematin; 0·3 ml. 1·4% denatured globin; 0·1M-phosphate buffer pH 7·3.)

Contents of manometric flasks	O_2 uptake/20 min. (μl.)
Oxid. + cyt. *c* + succin.	209
Oxid. + cyt. *c* + succin. + haematin	18
Oxid. + cyt. *c* + succin. + globin	188
Oxid. + cyt. *c* + succin. + globin + haematin	176

If succinate be replaced by *p*-phenylenediamine in order to measure cytochrome oxidase activity, the same concentration of haematin produces less than 25% inhibition. It is important to note that three haematins, namely proto-, deutero- and haematohaematin produce approximately the same inhibitory effect on the succinic system while Fe^{+++}, porphyrin and globin parahaematin (cathaemoglobin) have no effect on it.

Similar experiments with the pigeon breast-muscle preparation are summarized in Table 2 and show that although denatured globin does not activate the succinic system in this material it protects it from the inhibition by haematin.

The effect of haematin on the anaerobic oxidation of succinic acid

In order to determine whether the inhibitory effect of haematin was due to its reaction with succinic dehydrogenase and not with some other component of the system, experiments were carried out in vacuum tubes with methylene blue as hydrogen acceptor. Four tubes (i, ii, iii, iv) were filled with reagents as shown in Table 3. Tubes i and iii were normal Thunberg tubes with one hollow stopper whereas tubes ii and iv were provided with two hollow stoppers (Fig. 1, *A*, *C*). The results of these experiments clearly show that a marked inhibition of methylene blue reduction is obtained under anaerobic conditions and this inhibition is partly reversed by cyanide which has a high affinity for haematin. The efficiency of cyanide in these experiments is less marked than that of glyoxalin or denatured globin in aerobic experiments. This may be due to the fact that we are dealing here with a genuine reversibility of inhibition and not merely

* This phenomenon, which may also be observed with other proteins, will be considered in a separate paper.

Table 3. *The effect of haematin and cyanide on the anaerobic oxidation of succinic acid by heart muscle preparation (oxid.) in presence of methylene blue (MB)*

(Experiments carried out in Thunberg tubes (*B*) with two hollow stoppers (*A* and *C*); $T = 39°$. 0·5 ml. oxid.; 0·04 M-sodium succinate; 0·5 ml. 10^{-3} M-MB; 0·3 ml. 2×10^{-3} M-haematin in 0·1 N-NaOH *or* 0·3 ml. 0·1 N-NaOH. When KCN in solid form was added (5 mg.) in *C* an equivalent amount of H_2SO_4 (0·7 ml. 0·1-N) was added from *A*. Volumes made up to 4 ml. with 0·1 M-phosphate buffer pH 7·3 in *B*. After evacuation and warming to 39°, *C* was added to *B*, then *A* added at zero time.)

Tube no.	*A*	*B*	*C*	Reduction time (min.)
i	Succinate	Oxid. + MB + NaOH	—	2·25
ii	Succinate + H_2SO_4	Oxid. + MB + NaOH	5 mg. KCN	3
iii	Succinate	Oxid. + MB + haematin	—	25
iv	Succinate + H_2SO_4	Oxid. + MB + haematin	5 mg. KCN	8·5

with a protection of the enzyme against the inhibitory effect of haematin. The use of cyanide in aerobic experiments is precluded owing to its powerful inhibitory effect on cytochrome oxidase.

By placing the haematin in one stopper and methylene blue with succinate in the other stopper of a special vacuum tube it was possible to add haematin to the oxidase preparation after the complete removal of air. In such experiments a marked inhibition by haematin was still obtained, the reduction time being of the order of 25 min. This shows that O_2 is not necessary for the inhibition of the enzyme by haematin.

The aerobic experiments summarized in Table 1 show that although glyoxalin will protect the dehydrogenase from the effect of haematin it cannot reverse the effect once the haematin has been added to the oxidase preparation. It has been possible, however, to reverse the effect to a considerable degree if the heart-muscle preparation is incubated *in vacuo* with haematin and succinate and then treated with glyoxalin. If the mixture is now placed in a manometer a rapid O_2 uptake ensues, but if the succinate is omitted during the incubation and only added in the manometer flask, the preparation is found to be inactive. For this experiment four double-stoppered vacuum tubes (v, vi, vii and viii) are filled as shown in the left half of Table 4, evacuated and warmed to 39°. The haematin and glyoxalin are then added at intervals as indicated in the table and the tubes cooled and opened. Definite volumes containing equal quantities of oxidase are then withdrawn from the tubes and tested manometrically with excess of cytochrome *c* and succinate. The results of these experiments which are given in the right half of Table 4 show that incubation of the heart preparation with haematin in absence of succinate produces the usual strong inhibition (vii and viii) but when succinate is present (v and vi) the inhibition is largely abolished.

Haematin and other enzymes

The effect of haematin was also tested on lactic dehydrogenase and the oxidation of glucose by crushed baker's yeast, *d*-amino-acid oxidase of pig kidney, lactic dehydrogenase of heart muscle and glucose oxidase (notatin) of *Penicillium notatum*. In no case did haematin exert an appreciable inhibitory effect. In the case of the cruder enzyme preparations this may be due to the presence of excess of inactive proteins which, especially if denatured, would compete for the haematin. Thus the conclusion of Yamafuji, Fujii & Imagawa (1943), that the activity of preparations oxidizing succinate is unaffected by haematin, may arise from the presence of denatured protein in their enzyme preparations. On the other hand, a more comprehensive investigation with purer materials may show that certain other enzymes are as sensitive to haematin as is succinic dehydrogenase.

Table 4. *Protection of succinic dehydrogenase against the inhibitory effect of haematin by incubation with succinate and glyoxalin*

(4 ml. 1 : 50 dilution heart preparation (oxid.) in phosphate buffer (0·1-M, pH 7·3), 0·6 ml. 0·4 M-sodium succinate, 0·2 ml. 0·004 M-haematin in 0·1 N-NaOH or 0·2 ml. 0·1 N-NaOH. Thunberg tubes (*B*) with two hollow stoppers (*A* and *C*) evacuated and warmed to 39°. *A* added to *B* and incubated 25 min. at 39°; *C* then added. Tubes cooled and opened and contents used for manometric experiments.)

	Filling of Thunberg tubes			Filling of manometer flasks				
Tube no.	*A*	*B*	*C*	Volume taken from Thunberg tube	0·4 M-succin. (ml.)	1% cyt. *c* (ml.)	pH 7·3 buffer (ml.)	O_2 uptake in manometers (μl. O_2/10 min.)
v	Haematin	Oxid. + succin.	Glyoxalin	2·7	0·3	0·3	—	74
vi	NaOH	Oxid. + succin.	Glyoxalin	2·7	0·3	0·3	—	107
vii	Haematin	Oxid.	Glyoxalin	2·4	0·3	0·3	0·3	0
viii	NaOH	Oxid.	Glyoxalin	2·4	0·3	0·3	0·3	100

DISCUSSION

The inhibition by haematin of succinic dehydrogenase is of special interest on account of its apparent specificity and the fact that the activity of this enzyme *in vivo* is intimately bound up with that of the cytochrome system which is composed of haematin compounds.

There are three possible explanations of the inhibition: (1) Haematin, being a good oxidation catalyst, may irreversibly inhibit the dehydrogenase by oxidizing certain groups essential for its activity. (2) The two propionic acid side chains of haematin, being in close proximity, may enable it to behave as a competitive inhibitor in the same way as malonate. (3) A parahaematin may be formed by reaction between haematin and some Fe-binding group essential for the catalytic activity of the dehydrogenase.

The evidence against the oxidation hypothesis is that the inhibition is equally marked in both aerobic and anaerobic experiments and also that globin parahaematin (cathaemoglobin) and especially glyoxalin parahaematin, which are much more efficient oxidizing catalysts than free haematin, have no inhibitory effect. Opposing the second suggestion are the facts that there is no competition between haematin and succinate and that, although two propionic groups are present in mesoporphyrin, the latter produces no inhibition. On the other hand, the third suggestion, namely the formation of a co-ordination compound between the haematin Fe and some important functional groups of succinic dehydrogenase, appears to be supported by the following three considerations: (*a*) Whereas iron and porphyrin alone are inactive their compound, haematin, is a strong inhibitor of the dehydrogenase, (*b*) proto-, haemato- and deuterohaematin are equally effective, and (*c*) glyoxalin and denatured globin, which both form very stable parahaematin compounds, show marked protection against inhibition by free haematin. The marked sensitivity of succinic dehydrogenase to free haematin suggests that not only the cytochrome components, catalase and peroxidase, but all other forms of intracellular haematin exist as compounds with proteins.

SUMMARY

1. Free haematin in a concentration of 2×10^{-4}M inhibits almost completely the oxidation of succinic acid by highly active muscle preparations.

2. In this respect proto-, haemato- and deuterohaematin are equally effective though neither iron nor porphyrins alone produce a comparable effect.

3. When haematin is bound to nitrogenous substances such as glyoxalin or denatured globin, forming the corresponding parahaematin compounds, it loses its inhibitory property. Hence the prior addition of glyoxalin or denatured protein will protect the enzyme preparation from inhibition by haematin.

4. Since the oxidation of succinic acid is also inhibited under purely anaerobic conditions in presence of methylene blue as hydrogen acceptor and since the oxidation of *p*-phenylenediamine is very little affected, the inhibitory effect is due to a reaction between haematin and succinic dehydrogenase and not to any influence upon the cytochrome system.

5. The inhibition is probably due to the formation of a co-ordination compound between haematin iron and some groups in succinic dehydrogenase important for its activity.

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The Determination of Oestrogens in Human Pregnancy Urine. A New Method of Correcting for the Brown Colour Developed in the Kober Reaction by Non-oestrogenic Substances

By MARGARET F. STEVENSON and G. F. MARRIAN
Department of Biochemistry, University of Edinburgh

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The determination of oestrogens in human pregnancy urine by the Kober (1931) method is complicated by the fact that urinary oestrogen concentrates may contain substances which yield a brown colour in the reaction with the phenolsulphonic acid reagent. Since this brown-coloured material shows considerable absorption at the wave-length of the absorption band of the pink colour developed by oestrogens, the latter may be considerably overestimated. The problem of correcting for this interfering brown colour, or of eliminating without loss of oestrogen the substances which give rise to it has been approached by a number of workers in different ways.

Cohen & Marrian (1934) measured the red colour of the final reaction mixture in Lovibond units and then subtracted the red component of the residual brown colour after fading the oestrogen pink colour with H_2O_2. However, as was pointed out by Venning, Evelyn, Harkness & Browne (1937) and by Bachman & Pettit (1941), this method of correction is not satisfactory since treatment with H_2O_2 causes some fading of the non-oestrogen brown colour. Venning *et al.* (1937) showed that the brown-coloured material absorbs strongly at 420 mμ. and that the oestrogen pink-coloured substance, which shows an absorption maximum at 522 mμ., is nearly transparent at the former wave-length. By assuming that the spectrophotometric characteristics of the non-oestrogen brown colour are constant for different urines they were able to calculate from the measured absorption at 420 mμ. the absorption at 522 mμ. due to this brown colour. The validity of the basic assumption underlying this method of correction has, however, been questioned by Bachman & Pettit (1941).

Kober (1938), in the paper in which a modified oestrogen reagent (β-naphthol and H_2SO_4) was described, observed that the oestrogen pink colour developed with this reagent fades in the presence of acetone. This fading in the presence of acetone has been utilized by Jayle, Crépy & Judas (1943) in a new method for correcting for the brown colour. After allowing the Kober reaction mixture to stand in the dark for several hours in the presence of acetone, the residual brown colour was measured spectrophotometrically and the appropriate correction applied to the original measurement of the pink colour.

Bachman & Pettit (1941) attempted to avoid the necessity of a correction by eliminating from the oestrogen concentrates the substances which give rise to the brown colour in the Kober reaction. By separating the oestriol and the oestrone-oestradiol fractions and then purifying these fractions by distribution between various pairs of immiscible solvents they obtained products which gave little brown colour, as judged by absorption at 420 mμ., in the Kober reaction.

A somewhat similar approach to the problem has been made more recently by Stimmel (1946). The total ether-soluble phenolic material obtained from urine was separated into oestriol, oestrone, and oestradiol fractions by adsorption on Al_2O_3 and fractional elution with different methanol-benzene mixtures. The three fractions so obtained gave colours in the Kober reaction which showed little absorption at 420 mμ. Stimmel has suggested that the final coloured reaction mixture should be shaken with ethyl acetate before measurement of the colour. It was claimed that this procedure removed impurities without affecting the pink colour in the aqueous phase.

The object of the present work was to evolve a simplified method for determining the total oestrogen in human pregnancy urine which would be suitable for clinical investigations. In the course of this work it was observed that whereas the pink colour given by oestriol or oestrone in the Kober reaction can be completely faded by heating for 1·5 hr. in the boiling water-bath, the brown colour given by the ether-soluble phenolic fraction of human male urine is affected by this treatment only to a very small extent. On the basis of these observations a simple procedure for correcting for the non-oestrogen brown colour given by human pregnancy urine oestrogen concentrates in the Kober reaction has been worked out. This correction procedure which, in principle, has much in common with the one suggested by Jayle *et al.* (1943), is undoubtedly superior to the H_2O_2 method of Cohen & Marrian (1934) and because of its simplicity would seem to have certain advantages over the methods employed by others for the avoidance of, or correction for, the interfering brown colour.

EXPERIMENTAL

Hydrolysis and extraction of urine

A method of hydrolysis of urine based on that suggested by Smith & Smith (1935) has been employed. These workers added 15% (v/v) of conc. HCl to the urine, heated to boiling

and continued the boiling for 10 min. It was shown by Smith, Smith & Pincus (1938) that the yields of free oestrogen extractable from human pregnancy urine after such a hydrolysis were as high as those obtained after the less convenient method of Cohen & Marrian (1935). Two modifications have been made in the Smith & Smith procedure: (i) In order to ensure accurate timing of the hydrolysis and to minimize oxidative destruction of the oestrogens, the urine specimens have been brought to the boil before the addition of the acid. (ii) It has been found that the yield of free oestrogen, as determined by the Kober method, is not maximal after boiling for 10 min. with 0·15 vol. of conc. HCl. The data available, which will be published in detail later, indicate that maximal hydrolysis of the conjugated oestrogens in human pregnancy urine is attained by boiling with 0·15 vol. of conc. HCl for 30–40 min. In the majority of the experiments reported in this paper, therefore, the hydrolysis has been continued for 30 min.

Urine specimens were collected with toluene as preservative. In order to standardize the procedure as far as possible, 24 hr. specimens were all diluted to a volume of 2·5 l. before removing samples for hydrolysis and extraction. The procedure for hydrolysis and extraction was as follows: 100 ml. of the diluted urine was heated to boiling under reflux and, after the addition of 15 ml. of conc. HCl down the condenser, boiling was continued for the required time (see above). The hydrolyzed urine was rapidly cooled under the tap and then extracted once with 100 ml. and twice with 50 ml. portions of ether. The combined ethereal extracts were washed three times with 25 ml. portions of 5% (w/v) $NaHCO_3$ and the combined washings back-extracted once with 20 ml. of ether. The washed ethereal extract was combined with the back-extract and evaporated to dryness. The residue was warmed with *c.* 3 ml. of ethanol and 100 ml. of benzene added. This benzene solution was then extracted once with 50 ml. and twice with 25 ml. portions of N-NaOH. The combined NaOH extracts were acidified with 15 ml. of conc. HCl and extracted once with 100 ml. and twice with 50 ml. portions of ether. The ethereal extracts were washed twice with 20 ml. portions of 5% (w/v) $NaHCO_3$, and the washings back-extracted once with 20 ml. of ether. The combined ethereal extracts after washing three times with 20 ml. portions of water, were evaporated to dryness. The residue, consisting of the total ether-soluble phenolic fraction of the hydrolyzed urine, was dissolved in ethanol and suitable measured samples of the solution pipetted out for colorimetric assay.

Technique of the Kober reaction

The phenolsulphonic acid reagent was prepared according to the method of Cohen & Marrian (1934). The technique of colour development adopted was essentially similar to that described by Venning *et al.* (1937).

Measured samples of the solution of the urinary phenolic fraction, containing between 10 and 80 μg. of oestrogen, and equivalent to not more than 2%* of the 24 hr. urine specimen, were evaporated to dryness in a stream of air in test tubes *c.* 2 cm. in diameter, bearing graduation marks at 8 and 15 ml. To the dry residues 3 ml. portions of the Kober reagent were added and the tubes were heated in the boiling water-bath for 20 min. After cooling in an ice-salt freezing mixture 3·0 ml. of water were added to each tube and the contents thoroughly mixed. The tubes were then heated for 3 min. in the boiling water-bath, cooled to room temperature by immersion in water and finally made up to the 15 ml. mark with 10% (v/v) H_2SO_4. The intensities of absorption at *c.* 520 mμ. were measured on 7 ml. portions of the final solutions in a Spekker photoelectric absorptiometer using an Ilford 'spectrum green, no. 604' light filter.

The 8 ml. portions of the solutions remaining in the tubes were heated in the boiling water-bath for 1·5 hr. in order to fade the pink colour produced by the oestrogen. After cooling and making good the water lost by evaporation, the absorption at *c.* 520 mμ. was again measured. The amount of oestrogen (as oestriol) originally present in each tube was obtained by referring the difference between the initial and final absorptiometer readings to an oestriol calibration curve.

* When amounts of phenolic fraction equivalent to more than 2% of the 24 hr. urine specimen were used precipitates were sometimes formed during the subsequent fading procedure.

'Blank' determinations on human male urine

As a necessary preliminary to recovery experiments in which oestrone and oestriol were added to male urine a series of 'blank' determinations was carried out on the latter.

24 hr. urine specimens (preserved with toluene) were collected from a number of healthy adult men. Samples from each were hydrolyzed and extracted according to the procedure described above, and Kober reactions carried out on amounts of the phenolic fractions equivalent to 1% and/or 2% of the 24 hr. specimens. The results are shown in Table 1.

It will be seen that the brown colours developed underwent only small changes in absorption at *c.* 520 mμ. as a result of heating at 100° for 1·5 hr. These small changes, however, correspond to 'apparent oestrogen' contents (calculated as oestriol) varying from 0·0 to 0·7 mg./24 hr. and averaging *c.* 0·3 mg.

The significance of this small 'apparent oestrogen' content of male urine is difficult to assess. The variations in the values obtained for the same urine specimen and between the values obtained for different urines must be to some extent due to unavoidable small errors in the absorptiometer readings. Thus an error of only 0·004 in the difference between the initial and final readings would correspond to an error of 0·1 mg./24 hr. in the 'apparent oestrogen' value when the determination is carried out on the equivalent of 1% of the 24 hr. specimen. Nevertheless, since the small observed changes in absorption at *c.* 520 mμ. resulting from heating the solutions were all decreases, they cannot be due only to the multiplication of small absorptiometer reading errors. This small decrease in absorption at *c.* 520 mμ. may be due largely to changes in the reaction mixture other than that resulting from the

Table 1. *'Blank' determinations on normal human male urine*

Urine	Hydrolysis time (min.)	Fraction of 24 hr. urine specimen used (%)	Absorptiometer readings (spectrum green filter) Initial (*A*)	After heating for 1·5 hr. at 100° (*B*)	Oestriol equivalent of *A* − *B* (μg.)	'Apparent oestrogen' content of urine (mg./24 hr.)
A	10	1	0·089	0·087	0·5	0·05
	10	2	0·167	0·163	1·0	0·05
	10	2	0·220	0·212	2·0	0·10
B	10	1	0·116	0·099	4·5	0·45
	10	2	0·152	0·138	4·0	0·20
	10	2	0·150	0·139	3·0	0·15
B.C. (i)	10	1	0·097	0·080	5·0	0·50
	10	1	0·112	0·089	6·5	0·65
G.A. (i)	10	1	0·090	0·078	3·5	0·35
	10	1	0·068	0·059	2·5	0·25
	10	1	0·077	0·064	3·5	0·35
	10	1	0·066	0·058	2·0	0·20
R.J. (i)	10	1	0·123	0·102	6·0	0·60
	10	1	0·109	0·083	7·3	0·73
R.J. (ii)	10	1	0·098	0·098	0·0	0·0
	10	1	0·082	0·081	0·0	0·0
B.C. (ii)	30	2	0·147	0·141	1·5	0·08
	30	2	0·154	0·151	0·75	0·04
G.A. (ii)	30	1	0·046	0·038	2·0	0·20
	30	1	0·062	0·044	5·0	0·50
	30	2	0·101	0·081	5·5	0·28
	30	2	0·111	0·099	3·0	0·15
	30	2	0·108	0·102	1·5	0·08
	30	2	0·120	0·082	10·8	0·54
R.J. (iii)	30	2	0·158	0·157	0·0	0·00
M.G.	30	1	0·091	0·070	5·8	0·58
	30	1	0·101	0·084	4·5	0·45
G.J.	30	1	0·067	0·057	2·3	0·23
	30	1	0·064	0·052	2·8	0·28
	30	2	0·137	0·124	3·0	0·15
	30	2	0·137	0·120	4·0	0·20
K.W.	30	1	0·050	0·044	1·5	0·15
	30	1	0·049	0·043	1·5	0·15
	30	2	0·106	0·090	4·0	0·20
	30	2	0·098	0·098	0·0	0·00

fading of an oestrogen pink colour. However, the alternative possibility that the 'apparent oestrogen' values are in fact due to oestrogen cannot be entirely disregarded. Callow, Callow, Emmens & Stroud (1939) have recorded oestrogen contents for human male urine, determined by bioassay, of *c.* 30–40 i.u./l. These values give a rough average of 50 i.u. of oestrogenic activity per 24 hr. From the data given by Emmens (1939) it may be calculated that this amount of oestrogenic activity would be given by 0·05 mg. of oestrone or by 0·35 mg. of oestriol. Accordingly if it were assumed that most of the oestrogen in human male urine is oestriol the average value for the 'apparent oestrogen' content determined colorimetrically is in reasonable agreement with the average value determined by bioassay. However, although there are indications that oestriol normally occurs in human male urine (Pincus, 1945), there is no reason to believe that it is the predominant oestrogen present.

Recovery of oestrone and oestriol added to hydrolyzed human male urine

In order to assess the value of the 'fading' technique as a method of correcting for the non-oestrogen brown colour, a series of recovery experiments was made in which oestrone and/or oestriol in known amounts were added to suitable measured portions of acid-hydrolyzed 24 hr. specimens of human male urine. The oestrogen was added to the urine after hydrolysis rather than before in order to avoid complicating the issue by the losses due to destruction which are known to occur during the hydrolysis (cf. Cohen & Marrian, 1935). Since the object of these experiments was to compare the recoveries obtained by the 'fading' technique

with those obtained by the direct Kober method, rather than to determine the overall error of the whole procedure, this course seemed to be justifiable.

With increasing amounts of oestrogen the corrected and uncorrected recovery figures become closer to one another, and it will be apparent from the results that for urines containing more than

Table 2. *Recovery of oestrogen added to acid-hydrolyzed human male urine*

Added oestrogen (mg./24 hr.)			Fraction of 24 hr. urine sample used (%)	Absorptiometer readings		Recovery of oestrogen (%)	
Oestrone	Oestriol	Total as oestriol		Before fading (*A*)	After fading (*B*)	From (*A* − *B*)	From (*A*)
0·00	1·00	1·00	2	0·168	0·108	80	210
0·00	1·00	1·00	2	0·165	0·107	78	205
1·05	0·00	1·16	2	0·248	0·162	100	275
1·05	0·00	1·16	2	0·224	0·140	97	266
0·70	0·30	1·07	2	0·220	0·161	78	264
0·50	1·58	2·13	1	0·145	0·074	92	178
0·50	1·58	2·13	1	0·151	0·070	104	185
1·60	0·53	2·30	1	0·154	0·073	94	172
1·60	0·53	2·30	1	0·147	0·070	91	164
0·00	5·30	5·30	1	0·253	0·067	95	123
0·00	5·30	5·30	1	0·248	0·067	93	121
3·13	0·99	4·44	1	0·250	0·072	116	152
3·13	0·99	4·44	1	0·255	0·077	116	157
4·00	0·00	4·40	1	0·231	0·066	104	130
4·00	0·00	4·40	1	0·234	0·068	105	131
2·09	4·95	7·25	0·4	0·140	0·028	112	129
2·09	4·95	7·25	0·4	0·139	0·027	112	129
2·50	10·0	12·8	0·4	0·206	0·037	89	98
2·50	10·0	12·8	0·4	0·206	0·038	88	98
0·00	15·9	15·9	0·4	0·248	0·029	94	101
0·00	15·9	15·9	0·4	0·232	0·030	87	94
15·0	0·00	16·5	0·4	0·254	0·033	102	104
15·0	0·00	16·5	0·4	0·267	0·036	107	110
10·0	25·0	36·0	0·1	0·128	0·014	85	86
10·0	25·0	36·0	0·1	0·131	0·011	89	88
26·1	9·90	38·6	0·1	0·151	0·014	103	107
26·1	9·90	38·6	0·1	0·169	0·013	116	118
0·00	64·5	64·5	0·1	0·211	0·018	85	81
0·00	64·5	64·5	0·1	0·228	0·022	89	87
68·3	0·00	75·1	0·1	0·270	0·021	90	93
68·3	0·00	75·1	0·1	0·281	0·018	95	96

The results of these recovery experiments are shown in Table 2. It will be seen that when less than *c.* 10 mg. of oestrogen/24 hr. was present, necessitating the carrying out of the colour reaction on the equivalent of 1 % or more of the 24 hr. urine specimen, the recoveries obtained by the uncorrected Kober method were grossly high, whereas those obtained by the 'fading' technique were mostly within the range of 80–110 %. It is justifiable, therefore, to conclude that this new method of correcting for the non-oestrogen brown colour will be of some value in the analysis of urine obtained during early and middle pregnancy.

c. 15 mg. oestrogen/24 hr. a correction is hardly necessary.

Particular attention must be drawn to the recoveries obtained when *c.* 1 mg. of oestrogen/24 hr. was present. In view of the magnitude of the male urine 'blanks' these apparently good recoveries must be regarded as being fortuitous, and it must be assumed that the small unavoidable losses which occur during the extraction process were largely offset by the urine 'blank'. In this connexion the authors are of the opinion that little quantitative significance should be placed on results indicating less than *c.* 2 mg. oestrogen/24 hr.

SUMMARY

1. The pink solutions yielded by oestrone or oestriol in the Kober reaction with the phenolsulphonic acid reagent are completely decolorized after heating at 100° for 1·5 hr.

2. The brown solutions yielded by ether-soluble phenolic fractions of human male urine in the Kober reaction undergo little change, as judged by absorption at *c.* 520 mμ., when similarly treated.

3. On the basis of these observations a simple method has been developed of correcting for the interfering brown colour which is developed from non-oestrogenic substances in the determination of oestrogens in human pregnancy urine.

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The Influence of *l*-Ascorbic Acid on the Rupture of the Benzene Ring of *l*-Tyrosine Consumed in High Doses by Guinea-pigs

By H. A. PAINTER and S. S. ZILVA (Member of the Scientific Staff, Medical Research Council), *Lister Institute, London*

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Kotake, Matsuoka & Okagawa (1922) observed that when a massive dose (10 g.) of *l*-tyrosine was administered to adult rabbits the aromatic part of the *l*-tyrosine molecule was not fully metabolized, since *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acids were excreted in the urine. Recent work by Sealock & Silberstein (1939, 1940) and Levine, Marples & Gordon (1939, 1941*a*, 1941*b*) threw a new light on the rupture of the benzene ring of *l*-tyrosine by the animal organism. The former investigators found that when 0·5 g. or more of *l*-tyrosine was administered to guinea-pigs on a scorbutic diet, homogentisic, *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acids were excreted in the urine. When, however, *l*-ascorbic acid was administered in sufficiently high doses the excretion of these aromatic compounds ceased. Levine and his co-workers made a similar observation on infants. They found that premature infants on diets containing 5 g./kg. body weight/day or more of protein excreted *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic, but not homogentisic acids. On the other hand, full-term infants on a similar diet only excreted these aromatic amino-acids when they consumed 1 g./kg. body weight of either *l*-tyrosine or *l*-phenylalanine. In both cases the excretion of the aromatic amino-acids could be abolished by the consumption of *l*-ascorbic acid. These observations led to a tentative suggestion that *l*-ascorbic acid participated directly or indirectly in protein metabolism. The experiments described in this communication were primarily devised to test this hypothesis.

EXPERIMENTAL

Technique

Experimental animals and diet (see Penney & Zilva, 1946).

Collection of urine. The experimental animals were kept in metabolism cages and the urines were collected in tubes which, except in experiments of short duration, contained sufficient HCl to keep the urines acid to congo red. The urines to be tested for homogentisic acid, were collected under liquid paraffin.

Preparation of tissues for analysis. The animals were killed by stunning and bleeding and the blood was collected in oxalate. Metaphosphoric acid was used in the extraction of the tissues instead of trichloroacetic acid, as the latter interfered with some of the colorimetric determinations. The whole blood and the urine were precipitated with equal volumes of 10% metaphosphoric acid. The liver, muscle, and the small and large intestines, the latter after removal of the contents and subsequent washing with water and drying the tissue with filter-paper, were extracted twice with one part by weight of 8·5% metaphosphoric acid by grinding with sand and centrifuging. The kidney and stomach were similarly treated with 4 parts by weight of 5% metaphosphoric acid. The contents of the intestinal tract, to which were added any faeces present (although the latter contained only traces of hydroxyphenyl compounds), were diluted with the smallest practicable amount of water and extracted with $\frac{1}{3}$ vol. of 20% metaphosphoric acid. The metaphosphoric acid extracts of the large intestines and the liver were sometimes found to be turbid, but the extract could be cleared with small quantities of kieselguhr, a process which did not interfere with the analyses.

Determination of total hydroxyphenyl compounds. This was carried out by the method of Folin & Ciocalteu (1927) as modified by Medes (1932), except that a calibration curve was used instead of standards. All experimental values were expressed in terms of tyrosine.

Determination of p-*hydroxyphenylpyruvic acid.* This was carried out by the Penrose & Quastel (1937) test using a calibration curve, obtained from a solution of *p*-hydroxyphenylpyruvic acid in 2·5% metaphosphoric acid which was allowed to remain for 30 min. after being adjusted to pH 9·0 (see Penney & Zilva, 1943; Painter & Zilva, 1947). It is to be pointed out that this test, apart from being more specific in this instance, is also more delicate than the Millon test and that the 2:4-dinitrophenylhydrazone has been isolated from the urines of guinea-pigs on a scorbutic diet receiving an excess of *l*-tyrosine.

*Glycyl-*l*-tyrosine.* This was prepared by the method of Fischer (1904). Preparations obtained by a single precipitation with ethanol were used. The purity of these preparations was assessed by the Folin & Ciocalteu method and was found to be about 85%.

l-*Tyrosine.* The commercial product was used for dosing but for standardizing purposes it was purified by the method of Folin & Ciocalteu (1927).

The influence of the method of administering high doses on the degradation of l-*tyrosine by the guinea-pig*

In the majority of their experiments, Sealock & Silberstein incorporated the *l*-tyrosine in the basal diet. The substance was thus consumed intermittently. It seemed to us that the oral administration of *l*-tyrosine suspended in a little water and given in one dose might be more advantageous, and it was, therefore, decided to investigate the excretion of the aromatic degradation products of tyrosine in the two procedures.

One group of three guinea-pigs on a scorbutic diet received orally in one dose 0·5 g. of *l*-tyrosine suspended in water, and in a similar group the *l*-tyrosine was incorporated in the basal diet. In both groups the amounts of total hydroxyphenyl compounds and of *p*-hydroxyphenylpyruvic acid excreted in the urine during 24 hr. were determined. The daily quantity of *l*-tyrosine consumed by animals receiving it in the diet varied from 0·5 to 0·8 g., and the amounts of excretion products determined were recalculated for a uniform consumption of 0·5 g. In Fig. 1 the results are shown as the means

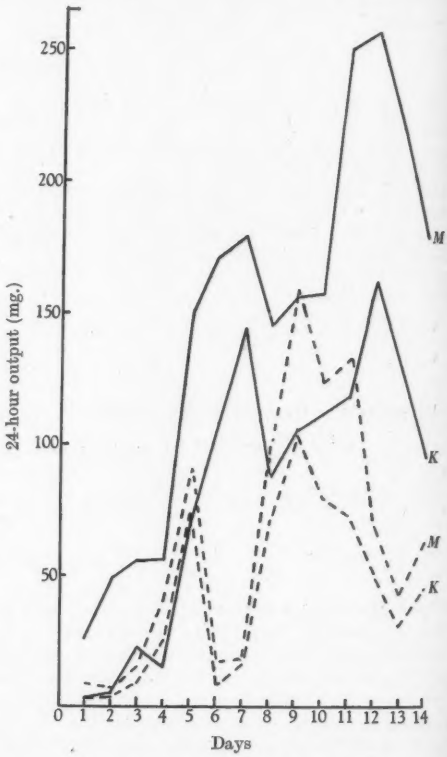

Fig. 1. Influence of the method of administration on the degradation of 0·5 g. of *l*-tyrosine by the guinea-pig. ——— Tyrosine administered in one single daily dose. – – – – Tyrosine added to basal diet. *M* = total hydroxyphenyl compounds. *K* = *p*-hydroxyphenylpyruvic acid.

for three animals. It will be seen that when the *l*-tyrosine was consumed in a single dose instead of intermittently a higher percentage of the tyrosine was excreted as aromatic compounds. For this reason we considered the single-dose technique more suitable for the investigation of our problem. In both cases the excretion of aromatic compounds commenced soon after the *l*-ascorbic acid was removed from the diet. When the *l*-tyrosine was given

in one dose the ratio K/M, i.e. *p*-hydroxyphenylpyruvic acid to total hydroxyphenyl compounds, was lower during the first 4 days of the *l*-ascorbic acid deprivation period, viz. $0{\cdot}23 \pm 0{\cdot}14$ for the first 4 days and $0{\cdot}61 \pm 0{\cdot}10$ for the next 10 days.

The influence of the magnitude of the dose on the excretion of aromatic intermediate products of l*-tyrosine degradation*

The aim of these experiments was to establish approximately the amounts of *l*-tyrosine which a guinea-pig on a scorbutic diet must consume, so that aromatic intermediate products of *l*-tyrosine degradation might appear in the urine. Single doses of 0·1, 0·2, 0·3, 0·4 and 0·5 g. of *l*-tyrosine were therefore administered orally to five groups of three guinea-pigs, 8 days and, again, 14 days after being put on a scorbutic diet. For comparison the amounts of total hydroxyphenyl derivatives and *p*-hydroxyphenylpyruvic acid excreted were determined for each animal on the day before the *l*-tyrosine was administered.

were deducted from the values obtained after administration of *l*-tyrosine. There was an increase in the excretion of *p*-hydroxyphenylpyruvic acid in only one of the guinea-pigs which received 0·1 g. of *l*-tyrosine after 8 days on a scorbutic diet, though there was a general small increase in the excretion of the total hydroxyphenyl derivatives on this dose. After 14 days of scorbutic diet there was a general increase in the excretion of both *p*-hydroxyphenylpyruvic acid and the total hydroxyphenyl derivatives in the group of animals given 0·1 g. of tyrosine. This increase was more marked in the animals receiving 0·2, 0·3 and particularly 0·4 and 0·5 g. of *l*-tyrosine after 8 or 14 days, and occurred in all the animals.

It would, therefore, appear that the proportion of *l*-tyrosine in which the benzene ring was ruptured *in vivo* became less with the rise in the dose. It is also to be noted that the benzene ring was opened in over 90 % of the *l*-tyrosine when the dose was 0·1 g., which, although a comparatively high dose, seems to be the border-line quantity for the excretion of aromatic intermediate products of degradation. In

Table 1. *The influence of the magnitude of the dose on the excretion of aromatic intermediate products of* l*-tyrosine degradation*

Days on scorbutic diet		0·1 g. *l*-tyrosine		0·2 g. *l*-tyrosine		0·3 g. *l*-tyrosine		0·4 g. *l*-tyrosine		0·5 g. *l*-tyrosine	
		K (mg.)	*M* (mg.)	*K* (mg.)	*M* (mg.)	*K* (mg.)	*M* (mg.)	*K* (mg.)	*M* (mg.)	*K* (mg.)	*M* (mg.)
8		0	12·6	4·6	70	61·0	84	43	63	—	—
		0	1·8	10·2	17	21·5	41	25	34	—	—
		3·0	4·2	81·0	127	24·0	35	107	159	—	—
	Mean	1·0	5·2	45·7	71·3	36·0	53	58·3	85	—	—
	S.D.	1·7	5·2	35·3	56	22·3	26·7	39·7	66	—	—
14		7·0	8·4	19·5	38	40·6	127	94	133	189	193
		1·7	3·1	16·0	21	46·5	63	138	180	62	110
		7·0	14·1	47·0	56	52·5	75	101	139	261	309
	Mean	5·2	8·5	27·5	38	46·5	88	111	151	171	204
	S.D.	3·0	5·5	16·5	17	6·0	34	23·6	25·6	102	100

In this and all the subsequent tables, K = *p*-hydroxyphenylpyruvic acid; M = total hydroxyphenyl compounds, calculated as tyrosine.

The results are incorporated in Table 1. The figures obtained before dosing revealed that there was some individual variation in excretion of substances which react with Millon's and Penrose & Quastel's reagents and that the continuance of the animals on a scorbutic diet for longer than 8 days did not significantly influence this excretion. The level of excretion in the control period was very low, viz. mean value for the total hydroxyphenyl compounds was 4·2 mg., S.D. 1·7 and for substances reacting with the Penrose & Quastel reagent 2·2 mg., S.D. 1·0 (calculated as *p*-hydroxyphenylpyruvic acid) and was of a similar magnitude to that obtained in some later experiments with guinea-pigs on a mixed diet containing cabbage. These 'blank' values

other words, the amount of *l*-tyrosine in the diet has to be abnormally high before a significant excess of these compounds can appear in the urine of guinea-pigs deprived of *l*-ascorbic acid, and even then the quantity excreted corresponds only to a part of the *l*-tyrosine consumed.

The dietary equivalent of the additional *l*-tyrosine is illustrated by the fact that guinea-pigs of the above weight consume about 25–30 g. of basal diet containing about 0·13 g. *l*-tyrosine and about 0·09 g. of *l*-phenylalanine. Consequently the extra consumption even of the border-line dose of 0·1 g. of *l*-tyrosine would necessitate the raising of the food intake to the region of 40 g. The average animal of this age would not consume this quantity willingly

and, in all probability, even if it were possible to administer such a quantity forcibly, it would not conduce to the animal's well-being.

The course of the differential degradation of high doses of l*-tyrosine by guinea-pigs deprived of* l*-ascorbic acid*

The object of this experiment was not only to investigate the excretion of the aromatic intermediate products of degradation but also to throw some light on the distribution of these products in the various tissues of the animals at varying intervals after the consumption of the *l*-tyrosine and to compare the results with those obtained with guinea-pigs on the same diet with the addition of cabbage as a source of *l*-ascorbic acid.

A dose of 0·5 g. of *l*-tyrosine was administered *per os* to the two groups of animals and the tissue, urine and intestinal contents were examined 3, 5 and 24 hr. after the administration of the dose. The results are given in Table 2. As the figures after 5 hr. were very similar to those obtained after 3 hr. they are omitted from the table for economy of space. It is seen that the ketonic substances (K) present in the tissues and in the urine of the control animals, although generally somewhat higher in the case of the animals which received cabbage, were of the same low order. These were not deducted from the results obtained after administration of *l*-tyrosine. Only traces of other hydroxyphenyl compounds ($M-K$) could be detected in the tissues of the control animals and these results are omitted from the tables.

The first point which emerges from these experiments is that if the total hydroxyphenyl compounds found in the tissues, in the intestinal contents, and in the urine be taken into consideration, the benzene ring was not ruptured after 3 hr. in 69 mg. (14 %) of the *l*-tyrosine dose in the case of the guinea-pigs receiving cabbage and in 255 mg. (51 %) in the case of animals receiving no *l*-ascorbic acid. After 24 hr. the two groups excreted 15 mg. (3 %) and 205 mg. (41 %), respectively, of these intermediate products of tyrosine degradation with the benzene ring intact and only traces were found in the tissues and in the intestinal contents (Table 5). Not only were there more intermediate degradation products found in the urine of animals not receiving cabbage, but also the ratio $K/(M-K)$ was found to be much higher, i.e. 3·02 as compared with 0·19 in animals receiving cabbage. It is not surprising that in the animals deprived of *l*-ascorbic acid less of the aromatic intermediate compounds were accounted for in the tissues and urine together after 3 or 5 hr. than after 24 hr., since the absorption of *l*-tyrosine from the intestinal tract and the excretion of the aromatic intermediate products were still in progress after 3 hr. (cf. Table 5). The distribution of *p*-hydroxyphenylpyruvic acid and of the other hydroxyphenyl compounds in the tissues shows that in the case of animals not receiving *l*-ascorbic acid these substances were significantly higher in the liver and in the muscle; in the kidney the *p*-hydroxyphenylpyruvic acid content only was higher. These observations unfortunately give no clear indication so far concerning the mechanism involved in the incomplete degradation of some of the *l*-tyrosine by the guinea-pigs deprived of *l*-ascorbic acid.

A striking feature of this experiment is the higher content of the total hydroxyphenyl compounds found 3 and 5 hr. after the administration of *l*-tyrosine in the large intestine of the animals deprived of *l*-ascorbic acid. The mean was 149 ± 60 mg. as compared with 21 ± 34 mg. in animals receiving cabbage. The statistical treatment of these data as described by Snedecor (1937) for the determination of t gave a t value of 4·59. This shows that the difference was significant.

In another experiment in which a daily dose of *l*-ascorbic acid instead of cabbage was given to 12 guinea-pigs, the quantities of total hydroxyphenyl derivatives found in the large intestine contents 3 hr. after the administration of 0·5 g. of *l*-tyrosine were compared with those in 12 animals receiving *l*-ascorbic acid in the form of cabbage *ad lib.* and in 12 animals on a scorbutic diet. It was found that the mean in the first case was 65 ± 32 mg., in the second 24 ± 29 mg. and in the third 159 ± 46 mg. The t values showed that the differences between all the groups were significant. The quantities of total hydroxyphenyl derivatives found in the urine of the animals of the above groups were 6·7, 8·9 and 40 mg. respectively, the difference in the case of the first two groups not being significant. This proves that the accumulation of total hydroxyphenyl derivatives in the large intestine of guinea-pigs on a scorbutic diet is due to the absence of *l*-ascorbic acid *per se* from the diet and that this accumulation is accompanied by a higher elimination of the substances from the kidney. Another point that emerges from this experiment is that there is a greater accumulation in the large intestine when the *l*-ascorbic acid is administered in solution than when it is given in the form of cabbage. This is probably due to the fact that in the former case the *l*-ascorbic acid passes through the intestine more quickly than in the latter case.

The parenteral production of aromatic intermediate products of l*-tyrosine degradation by guinea-pigs subsisting on a scorbutic diet*

The observation that there was a higher content in the large intestine of hydroxyphenyl compounds 3–5 hr. after the ingestion of 0·5 g. of *l*-tyrosine in the case of guinea-pigs not receiving *l*-ascorbic acid,

Table 2. *The distribution of hydroxyphenyl compounds in the body and urine of guinea-pigs maintained on diets with and without* l*-ascorbic acid after administration of* 0·5 *g.* l*-tyrosine*

		Mixed diet containing cabbage						Scorbutic diet for 14 days									
		Control, no tyrosine administered, 3 animals *K*		3 hr. after *l*-tyrosine administration, 5 animals				Control, no tyrosine administered, 3 animals *K*		3 hr. after *l*-tyrosine administration, 6 animals				24 hr. after *l*-tyrosine administration, 4 animals			
				M − *K*		*K*				*M* − *K*		*K*		*M* − *K*		*K*	
		Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)
Stomach tissue:	Mean	Trace	Trace	1·9	56	Trace	Trace	Trace	Trace	2·0	67·3	Trace	Trace	Trace	Trace	Trace	Trace
	S.D.	—	—	0·8	—	—	—	—	—	0·8	—	—	—	—	—	—	—
Stomach contents:	Mean	0·2	—	6·6	—	0·23	—	Trace	—	7·0	—	Trace	—	Trace	—	0·1	—
	S.D.	0·0	—	5·0	—	0·3	—	—	—	7·7	—	—	—	—	—	0·1	—
Small intestine tissue:	Mean	0·23	2·2	3·6	30·8	0·14	1·2	Trace	Trace	5·2	38·8	0·15	1·2	Trace	Trace	0·16	1·4
	S.D.	0·06	—	2·8	—	0·09	—	—	—	2·1	—	0·1	—	—	—	0·06	—
Small intestine contents:	Mean	0·12	—	2·6	—	0·4	—	Trace	—	2·4	—	0·35	—	Trace	—	0·16	—
	S.D.	0·13	—	1·5	—	0·2	—	—	—	0·8	—	0·2	—	—	—	0·2	—
Large intestine tissue:	Mean	0·11	1·0	3·2	35·6	0·14	1·4	0·06	0·51	3·9	37·6	0·18	1·6	Trace	Trace	Trace	Trace
	S.D.	0·07	—	2·6	—	0·1	—	0·01	—	1·7	—	0·11	—	—	—	—	—
Large intestine contents:	Mean	1·5	—	20·0	—	1·1	—	0·34	—	147·6	—	1·6	—	Trace	—	1·6	—
	S.D.	0·9	—	34·0	—	0·9	—	0·07	—	59	—	1·4	—	—	—	1·4	—
Blood:	Mean	0·17	0·8	7·2	35·9	0·53	2·6	Trace	Trace	10·8	53·3	0·77	3·8	Trace	Trace	Trace	Trace
	S.D.	0·05	—	4·5	—	0·3	—	—	—	3·5	—	0·5	—	—	—	—	—
Liver:	Mean	0·33	3·4	2·4	23·2	0·34	2·8	0·39	2·8	4·7	37·4	0·53	4·2	Trace	Trace	0·76	5·2
	S.D.	0·22	—	2·1	—	0·19	—	0·08	—	1·2	—	0·25	—	—	—	0·18	—
Kidney:	Mean	0·09	3·9	1·6	65·2	0·08	3·8	0·05	1·5	1·8	68·7	0·20	7·0	Trace	Trace	0·07	2·7
	S.D.	0·1	—	1·6	—	0·07	—	0·01	—	0·8	—	0·08	—	—	—	0·04	—
Muscle:	Mean	1·6	3·3	5·9	11·8	0·55	1·1	1·0	1·7	27·2	52·5	2·5	4·5	Trace	Trace	0·55	1·0
	S.D.	0·1	—	5·1	—	0·15	—	0·9	—	13·0	—	3·0	—	—	—	0·30	—
Urine:	Mean	0·25	—	9·6	—	1·5	—	0·33	—	14·3	—	21·8	—	51·0	—	154	—
	S.D.	0·1	—	10·0	—	1·1	—	0·5	—	13·0	—	12·8	—	25·0	—	82	—

as compared with those which did, made it necessary to ascertain whether the opening of the benzene ring in the excess *l*-tyrosine took place parenterally and not solely in the intestine. In order to settle this point the urinary excretion of guinea-pigs receiving the scorbutic diet with and without the addition of cabbage was investigated after the injection of a series of single graded doses of glycyl-*l*-tyrosine. The urines were examined (*a*) from the animals which were maintained on the basal diet with cabbage *ad lib.* for 6 days previous to the injection, (*b*) after they were deprived of cabbage for 8 days, (*c*) after a further 5 days on the *l*-ascorbic acid deficient diet and finally, (*d*) 2 days after cabbage was reintroduced into the diet. The dipeptide was injected intraperitoneally. The urinary excretion of the aromatic products of degradation was found to be complete 24 hr. after injection and the urine was analyzed then.

It will be seen from Table 3 that there can be little doubt that the opening of the benzene ring can take place, at least in the main, outside the intestinal tract and that the mechanism of this change is similar whether the *l*-tyrosine is introduced orally or parenterally. These experiments also bring out the fact that when the dose of *l*-tyrosine is not too high, viz. 0·3 g., the abnormal degradation becomes evident only after the guinea-pig organism has been more thoroughly depleted of ascorbic acid, i.e. after 13 days on the scorbutic diet; with the higher doses this phenomenon was already to be observed after 8 days of *l*-ascorbic acid depletion. We are inclined to believe that this also operates when the *l*-tyrosine is consumed orally, but owing to the large individual variation and the comparatively few animals involved in all these experiments it cannot be demonstrated statistically every time. Also in these experiments an increase in the excretion of total hydroxyphenyl compounds by the ascorbic acid deficient animals was accompanied by an increase in the excretion of *p*-hydroxyphenylpyruvic acid. The border-line dose in these experiments seems to be somewhat higher than that found in the experiments when the *l*-tyrosine was administered orally. Another deviation in the above results from those obtained with the animals which received the *l*-tyrosine orally, namely that the quantity of the total hydroxyphenyl compounds excreted was higher when the 0·5 g. of *l*-tyrosine was injected (cf. Table 1). This is in all probability due to the fact that the amount of *l*-tyrosine circulating as the soluble dipeptide, or even as degradation products of *l*-tyrosine, in the body in the early stages after the injection, is much higher than that present at any time when a single dose is consumed orally and this in consequence leads to a more intense clearance by the kidney. This view is supported by the fact that the increased excretion has also been observed when

Table 3. *The parenteral production of aromatic intermediate products of l-tyrosine degradation by guinea-pigs subsisting on a scorbutic diet*

Animal no.	Equivalent *l*-tyrosine injected (g.)	Scorbutic diet + cabbage (6 days)				Scorbutic diet (8 days)				Scorbutic diet (13 days)				Scorbutic diet + cabbage (2 days)			
		M		*K*		*M*		*K*		*M*		*K*		*M*		*K*	
		(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)
1	0·55	53	9·7	6	1·1	189	34·2	108	19·6	282	51·3	174	31·5	64	11·6	6	1·1
2	0·58	40	7·0	4	0·7	342	59·0	142	24·5	336	57·8	177	29·5	30	5·2	13	2·1
3	0·29	50	18·0	5	1·7	15	5·1	2	0·7	131	45·0	62	21·3	34	11·8	5	1·7
4	0·29	31	10·6	2	0·7	36	12·4	3	0·9	145	49·9	70	24·1	14	4·9	2	0·6
5	0·29	33	11·4	1·5	0·5	49	16·9	7	2·2	109	37·5	41	14·1	29	10·0	4	1·3
6	0·29	40	13·7	4	1·3	15	5·1	3	0·9	34	11·7	4	1·3	29	10·0	3	1·0
7	0·17	21	12·1	2	1·2	15	8·7	2	1·2	24	13·8	3	1·7	8·0	4·6	1	0·5
8	0·17	33	19·1	3·5	2·0	19	10·9	2	1·2	16	9·2	3	1·7	17	9·9	4	2·1
9	0·18	12	6·7	2	1·1	18	10·0	3	1·6	11	6·1	2	1·1	30	16·7	3	1·5
10	0·06	9	16·3	2	3·5	7	12·7	2	3·5	10	18·1	2	3·5	14	25·4	3	5·1
11	0·06	4	7·2	2	3·5	9	16·3	2	3·5	6	10·9	3	5·1	19	34·5	3	5·1

the animals received cabbage. It will be noticed that when the guinea-pigs, after being depleted of *l*-ascorbic acid for 13 days, were offered cabbage for 48 hr. their capacity for dealing with high doses of *l*-tyrosine became similar to that observed in the preliminary cabbage period. In other words, their ability for opening the benzene ring in the entire dose of *l*-tyrosine was restored immediately on becoming 'supersaturated' with *l*-ascorbic acid (cf. Penney & Zilva, 1946). Experiments after prolonged cabbage feeding showed that once circulation of the excess of *l*-ascorbic acid was attained no further change occurred in the degradation of *l*-tyrosine within the limits of the doses employed.

It may be mentioned here that an attempt was made to ascertain whether the injection of much higher doses of *l*-tyrosine in guinea-pigs receiving cabbage *ad lib.* would bring about an abnormal excretion of the total hydroxyphenyl compounds, particularly *p*-hydroxyphenylpyruvic acid. Unfortunately we encountered certain technical difficulties in the attempt. An indication was however obtained that it may be so. In the only successful experiment, in which 2 g. of glycyl-*l*-tyrosine was injected, an excretion of total hydroxyphenyl compounds equivalent to 25 % and of *p*-hydroxyphenylpyruvic acid equivalent to 5 % of the injected *l*-tyrosine was found in the urine. These percentages, although lower than those which were found in the case of animals subsisting on a scorbutic diet were significantly above those so far observed in animals receiving cabbage *ad lib.* and injected with smaller doses of glycyl-*l*-tyrosine.

The absence of homogentisic acid from the urines of guinea-pigs receiving high doses of tyrosine on a scorbutic diet

In view of the fact that Sealock & Silberstein (1940) established the presence of homogentisic acid in the urine of guinea-pigs receiving *l*-tyrosine on a diet free of vitamin C, we tested the urine of some of our experimental animals for the presence of this acid. Tests were carried out on urines obtained from a group of six guinea-pigs maintained on a scorbutic diet for 14 days and receiving a daily dose of 0·5–0·8 g. of *l*-tyrosine orally, and also from a group of three animals which received 0·5 g. of *l*-tyrosine on three consecutive days and 1·0 g. on the fourth day, after previously subsisting on a scorbutic diet for 8 days and during the four subsequent days when dosed. As the coloration with ferric chloride and the reduction of ammoniacal silver nitrate are also given by *p*-hydroxyphenylpyruvic acid, it was not surprising that these tests were given by the urines of all animals receiving *l*-tyrosine on the scorbutic diet. On the other hand, the reaction with neutral silver nitrate and the darkening on aeration of the alkaline urines which are characteristic of homogentisic acid, were not observed, nor could a benzoyl derivative be isolated. On addition of 25 mg. of pure homogentisic acid, kindly supplied by Prof. Claude Rimington, to 10 ml. of a control urine, however, the characteristic reactions for the acid were observed in the above tests. No attempt was made to isolate the benzoyl derivative in this case. The above reactions were also negative in extracts of the intestinal contents and of the liver of guinea-pigs on a scorbutic diet 3 hr. after receiving a dose of 0·5 g. of *l*-tyrosine. Also, contrary to Sealock's observations, the quantities of *p*-hydroxyphenylpyruvic acid found by the Briggs reaction in the urines of three of our animals in which the comparison was made corresponded closely to those given by the Penrose & Quastel test and consequently did not indicate the presence of homogentisic acid.

DISCUSSION

The results obtained in these experiments leave little doubt that the excretion of hydroxyphenyl compounds by guinea-pigs receiving high doses of *l*-tyrosine after *l*-ascorbic acid has been withdrawn from the diet, is in the main due to an abnormality in the degradation of the excess of this compound by the general system, since this excretion was observed whether the *l*-tyrosine was administered orally or parenterally. Furthermore, this abnormal excretion was characterized by the presence of *p*-hydroxyphenylpyruvic acid in the urine in both cases.

If all the experimental evidence be brought into focus two factors appear to dominate the problem. In the first place very high doses of *l*-tyrosine must be consumed by guinea-pigs on the scorbutic diet before the failure to rupture the benzene ring in a part of the ingested compound can be observed. So much is this the case, that, in order to ensure the consumption in the form of a natural diet even of the border-line dose of 0·1 g. of *l*-tyrosine, which is just capable of producing the abnormal excretion, guinea-pigs must eat quantities of food so large as to make likely a deleterious effect on their health. The second outstanding point to emerge from the experimental results is that this abnormal degradation of high doses of *l*-tyrosine becomes effective very soon after the *l*-ascorbic acid is removed from the diet. Sealock & Silberstein (1940) found the maximum excretion of hydroxyphenyl compounds to occur as early as 24–48 hr. after the animals were deprived of the vitamin. In our experiments also the excretion of these compounds was observed to begin as early as the first day, although the maximum was not reached until a few days later (Fig. 1). In other words, rupture of the benzene ring does not take place in the whole of the *l*-tyrosine if the dose is high even when the guinea-pig tissues are almost

entirely saturated with *l*-ascorbic acid. The following considerations illustrate the point. Penney & Zilva (1946) have shown that the tissues of guinea-pigs are 'supersaturated' during the first few hours after the removal of cabbage from the animals' diet, owing to the 'flushing' of the saturated tissues by the excess of *l*-ascorbic acid still circulating in the system following the absorption of considerable quantities of *l*-ascorbic acid from the cabbage. In Table 4 the

Table 4. *Percentage loss of vitamin C in small intestine after* l*-ascorbic acid depletion*

No. of days depleted	Percentage loss calculated on supersaturated value	Percentage loss calculated on saturated value
1	35·8	6·5
2	51·0	28·6
3	60·8	42·8
4	66·7	51·5
5	72·6	60·0
6	77·4	67·2

losses of *l*-ascorbic acid (calculated as percentages of the initial 'supersaturated' ascorbic acid concentration, as well as of the theoretical saturated values) in the small intestine, which is representative of most of the other tissues, are given for the first few days after the removal of cabbage from the diet. It is seen from these figures that the abnormal degradation of part of the *l*-tyrosine, when administered in a dose as high as 0·5 g. daily, begins almost simultaneously with the disappearance of the excess of *l*-ascorbic acid which 'flushes' the tissues and at a time when the organism is almost fully saturated, i.e. 93·5%, and that at this failure to break down the benzene ring in a part of the dose which reaches a maximum at 3–4 days, only about a half of the *l*-ascorbic acid which saturated the body has disappeared. In addition we have found an indication that when the dose is high enough this abnormality may be observed even when the animal is 'supersaturated'. It is not surprising therefore that Kotake *et al.* (1922) observed that when large quantities of *l*-tyrosine were administered to rabbits, animals capable of synthesizing *l*-ascorbic acid in sufficient quantities for normal metabolic purposes, they excreted *p*-hydroxyphenylpyruvic acid and were unable to rupture the benzene ring in the whole administered *l*-tyrosine when the dose was excessive. Other examples of how abnormal consumption of aromatic amino-acids may lead to an excretion of intermediate products of degradation are (*a*) an experiment performed by Abderhalden (1912) in which a subject, though not alkaptonuric, excreted homogentisic acid after the consumption of 50 g. of *l*-tyrosine *per os*, and (*b*) one by Papageorge & Lewis (1938) who induced alkaptonuria in normal rats by the administration of high doses of *l*-phenylalanine. We therefore do not consider that the necessity for the presence of an excess of *l*-ascorbic acid in the guinea-pig system in order that the benzene ring shall be ruptured in all the *l*-tyrosine consumed when the dose is abnormally high, is an indication of the existence of a connexion between the normal function of *l*-ascorbic acid and the normal metabolism of aromatic, and still less other, amino-acids. The apparent interaction *in vivo* between *l*-ascorbic acid and *l*-tyrosine under these conditions suggests to us a physiological response to an unusual situation.

The observation made in the course of this inquiry that large quantities of hydroxyphenyl compounds are found 3 hr. after the ingestion of 0·5 g. of *l*-tyrosine in the large intestine of guinea-pigs which have been existing on a scorbutic diet for some days, is not without interest. For convenience the results given in Table 2 are summarized in Table 5. It will

Table 5. *Summary of the distribution of hydroxyphenyl compounds in the body and urine of guinea-pigs maintained on diets with and without* l*-ascorbic acid after the administration of* 0·5 *g.* l*-tyrosine; obtained from Table* 2 *(mg.)*

	Mixed diet containing cabbage		Scorbutic diet for 14 days	
	3 hr. after tyrosine dose	24 hr. after tyrosine dose	3 hr. after tyrosine dose	24 hr. after tyrosine dose
Tissues	27	Trace	60	Trace
Large intestine contents	21·1	Trace	149·1	Trace
Remainder of intestinal tract	9·8	Trace	9·7	Trace
Urine	11·1	15	36	205
Total	69	15	254·8	205

be seen from this summary that 3 hr. after the administration of *l*-tyrosine only 69 mg. of hydroxyphenyl compounds out of the 500 mg. of *l*-tyrosine administered could be accounted for in the case of guinea-pigs receiving cabbage. These compounds were distributed amongst the various tissues, the intestinal tract, and the urine excreted during the 3 hr. After 24 hr. the hydroxyphenyl compounds had disappeared from the body of animals receiving the same diet, having apparently been degraded in the system, since the quantity present in the urine was of the same order as that found after 3 hr. In the case of animals which did not receive cabbage the position was different. An equivalent of about as much as a half of the *l*-tyrosine dose could be accounted for after 3 hr., and 149 mg. of this was still present in the large intestine although the content in the remainder of the intestinal canal was of the same order as that observed in the case of

animals receiving cabbage. That most of these hydroxyphenyl compounds could enter the blood stream and be excreted without the rupture of the benzene ring to any great extent is seen from the fact that as much as 205 mg., found entirely in the urine, could be accounted for after 24 hr. in a similar group of animals not receiving cabbage. The hypothesis that the *l*-tyrosine in the large intestine was broken up by bacteria before it entered the system to a greater extent when the guinea-pigs were consuming *l*-ascorbic acid than when they were deprived of it was put to the experimental test. Guinea-pigs receiving cabbage were dosed with sulphaguanidine for several days previous to receiving the *l*-tyrosine dose in order to sterilize partially the contents of the large intestine. It was found that after this treatment there was indeed an accumulation of hydroxyphenyl compounds in the large intestine similar to that found in the case of the guinea-pigs which were deprived of *l*-ascorbic acid but, unlike the latter animals, they did not excrete significant amounts of these compounds in the urine even after 24 hr. It is evident that this problem calls for further inquiry.

SUMMARY

1. Guinea-pigs deprived of *l*-ascorbic acid and receiving 0·5 g. or more of *l*-tyrosine daily, excreted in the urine hydroxyphenyl compounds which included *p*-hydroxyphenylpyruvic acid. The excretion began soon after the removal of *l*-ascorbic acid and reached a maximum in 4–5 days.

2. When the *l*-tyrosine was consumed in a single daily dose instead of being ingested intermittently in the basal diet, more hydroxyphenyl compounds were excreted, but the proportion of *p*-hydroxyphenylpyruvic acid was lower during the first 4 days after the *l*-ascorbic acid was removed from the diet.

3. The total hydroxyphenyl compounds and the *p*-hydroxyphenylpyruvic acid excreted by guinea-pigs deprived of *l*-ascorbic acid increased as the dose of *l*-tyrosine increased. The abnormal excretion became just noticeable when the dose was 0·1 g.

4. The examination of the tissues, intestinal contents, and urine of guinea-pigs, on a scorbutic diet with and without cabbage, 3, 5 and 24 hr. after the oral administration of 0·5 g. of *l*-tyrosine gave no clear indication of the mechanism involved in the incomplete degradation of *l*-tyrosine. After 3 or 5 hr. the proportion of *l*-tyrosine no longer recognizable as hydroxyphenyl compounds was 49% in animals receiving no *l*-ascorbic acid and 86% in those receiving cabbage, as compared with 59 and 97%, respectively, after 24 hr. The ratio of *p*-hydroxyphenylpyruvic acid to total hydroxyphenyl compounds excreted was much higher in animals not receiving *l*-ascorbic acid.

5. Similar results were obtained when *l*-tyrosine was administered parenterally.

6. Very much larger quantities of hydroxyphenyl compounds were found in the large intestine of guinea-pigs 3 or 5 hr. after ingestion of 0·5 g. of *l*-tyrosine when they were deprived of *l*-ascorbic acid than when they received the vitamin. 24 hr. after the consumption of the *l*-tyrosine none of these compounds could be found in the large intestine, but there was a marked increase in the urine. No significant increase was observed after 24 hr. in the urine of guinea-pigs receiving cabbage.

7. Homogentisic acid could not be found in the urine even after continued daily doses of 0·5–0·8 g. of *l*-tyrosine to guinea-pigs on a scorbutic diet.

8. The dependence of complete degradation of *l*-tyrosine in high doses on the presence of excess *l*-ascorbic acid in the guinea-pig system does not necessarily indicate a connexion between the normal function of *l*-ascorbic acid and the normal metabolism of aromatic amino-acids.

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The Tautomeric Conversion of *p*-Hydroxyphenylpyruvic Acid

By H. A. PAINTER and S. S. ZILVA (Member of the Scientific Staff, Medical Research Council)
Lister Institute, London

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It is well known that *p*-hydroxyphenylpyruvic acid in aqueous solution undergoes tautomeric change. According to Bougault & Hemmerlé (1915) the chemically related phenylpyruvic acid exists in the solid state in the enolic form, but in aqueous solution it is gradually converted into the ketonic tautomer. In our attempt to prepare standards for the determination of *p*-hydroxyphenylpyruvic acid in connexion with another investigation (Painter & Zilva, 1947) we soon realized that a similar change was taking place in the case of this acid, since the colour given by the Penrose & Quastel (1937) keto test became more intense as time progressed, whilst at the same time the indophenol titration values, indicating the amount of the enolic form, fell. This rendered it advisable to study the tautomeric conversion in some detail in order to ensure a more intelligent interpretation of the results obtained in our main inquiry. As will be seen, various observations were made in this connexion which invited further investigation but these were not pursued as they had no bearing on our immediate purpose.

METHODS

Reagents. (1) *p*-Hydroxyphenylpyruvic acid. The acid was prepared according to the method of Neubauer & Fromherz (1910) as modified by Raper & Wormall (1925). The standard solutions of *p*-hydroxyphenylpyruvic acid (0·01%, w/v) were made up immediately before use.

(2) 2:4-Dinitrophenylhydrazine reagent, see Penney & Zilva (1943).

(3) N-NaOH (A.R.).

Methods. The Penrose & Quastel test was carried out by diluting 5 ml. of the test solution to 10 ml. with distilled water. 1 ml. of the hydrazine solution was then added and the mixture shaken. The reaction was allowed to proceed for 30 min. at room temperature, after which time 5 ml. of N-NaOH were added and the solution diluted further to 25 ml. with distilled water. On the addition of the NaOH a brown colour was produced which rapidly disappeared. The measurement of the permanent red colour was carried out as described by Penney & Zilva (1943).

The Briggs test was carried out according to the procedure of Medes (1932). Our standard solution, however, was prepared by dissolving *p*-hydroxyphenylpyruvic acid in normal guinea-pig urine and was allowed to stand for 1 hr. The solution was then diluted 1 in 10 and after the addition of the reagents the colour was measured by the photoelectric colorimeter after 5 min.

RESULTS

Effect of pH *on the tautomeric conversion of p-hydroxyphenylpyruvic acid in aqueous solution.* These results are summarized in Fig. 1, from which it will

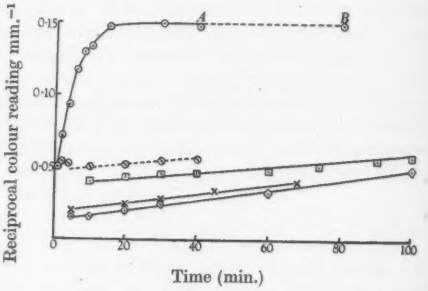

Fig. 1. Effect of pH on enol-keto conversion in aqueous solution at 15°. ⊙—⊙ pH 9·0, adjusted in this and all subsequent experiments with 0·01 N-NaOH. ⊘—⊘ Brought to pH 1·0 with HCl immediately after adjusting to pH 9·0 with 0·01 N-NaOH. ⊡—⊡ pH 7·4, adjusted with 0·01 N-NaOH. ×—× pH 4·0, aqueous solution of the keto acid. ◇—◇ pH 1·0, adjusted with HCl. (*A*) Solution adjusted to pH 1·0 at this point. In this and all subsequent experiments each test solution contained 0·5 mg. of solid *p*-hydroxyphenylpyruvic acid.

be seen that the change from the enolic to the ketonic form proceeds rapidly in alkaline solution (pH 9·0) and very slowly in acid and neutral media. At pH 7·4 the velocity of the conversion is of the same order as that at lower pH values. Although the points on the curves in Fig. 1 are those of the ketonic tautomer as determined by the Penrose & Quastel reaction, corresponding points could be obtained when the enolic form was determined in the same solution by titration with dichlorophenolindophenol and the results subtracted from the total content, thus confirming the values (Fig. 2). The results obtained by the Penrose & Quastel reaction can lay claim to higher accuracy since there was a lag in the end-point of the indophenol titrations and consequently figures for the enolic form tended to be on the low side. It may be pointed out that whilst the indophenol titration indicated the presence of

only 1–2 % of the enolic form at pH 9·0, when the solution was adjusted to pH 1·0 and allowed to stand for several days this value was raised to 10 %. The reconversion is evidently slow since it could not be demonstrated after 40 min. (see the interrupted line in Fig. 1, *A* to *B*). When however the solution at pH 9·0 was brought up immediately to pH 1·0 by the addition of HCl, bringing it up approximately to the pH under which the Penrose & Quastel test is carried out, the rate of conversion was simultaneously reduced to that which is characteristic for this particular pH.

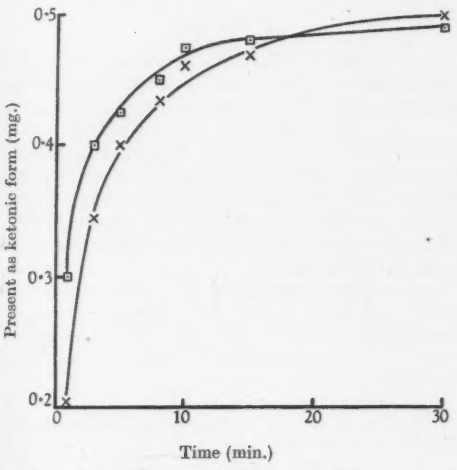

Fig. 2. Enol-keto conversion at pH 9·0 at 12°. ⊡—⊡ By indophenol titration. ×—× By the Penrose & Quastel test.

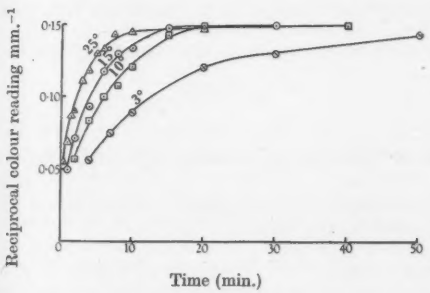

Fig. 3. Effect of temperature on enol-keto conversion at pH 9·0 as determined by the Penrose & Quastel reaction. △—△ 25°. ⊙—⊙ 15°. ⊡—⊡ 10°. ⊘—⊘ 3°.

Effect of temperature on the tautomeric conversion of p-*hydroxyphenylpyruvic acid at* pH 9·0. It is seen from Fig. 3 that, as was to have been expected, the rate of conversion increases with increasing temperature and that experiments can conveniently be carried out at room temperature. It was further found that when the log. of the percentage of the enolic tautomer present was plotted against the time the solution had stood, the curves approximated to straight lines thus showing that it was a reaction of the first order.

Tautomeric conversion of p-*hydroxyphenylpyruvic acid in urine and tissues.* Crystalline *p*-hydroxyphenylpyruvic acid was added to normal undiluted guinea-pig urine which was previously adjusted to pH values of 4·0, 7·4 and 9·0, and the acid was then determined by the Penrose & Quastel reaction after 30 min. At the two highest pH values the maximum formation of the ketonic form was already reached by that time. At pH 4·0 the maximum conversion value was reached after 100 min., i.e. at a much higher rate than in aqueous solution in which the maximum value was reached only after 1–2 days (see Fig. 1). It is, therefore, evident that the conversion from the enolic to the ketonic tautomer proceeds at a very much higher rate in neutral and acid urines than it does in the corresponding aqueous solutions and consequently *p*-hydroxyphenylpyruvic acid may usually be expected to be present in the ketonic form.

In another experiment when the blood, liver, urine, stomach and large intestine contents from guinea-pigs, in which *p*-hydroxyphenylpyruvic acid was expected on dietetic grounds to be present, no difference in the Penrose & Quastel values could be observed when the metaphosphoric acid extracts of the material and the untreated urine were adjusted to pH 4·0 and 9·0. It would appear that *p*-hydroxyphenylpyruvic acid is also present in the tissues as the ketonic tautomer.

In view of the fact that the Briggs reaction is very often used in the determination of *p*-hydroxyphenylpyruvic acid it was advisable to compare the results obtained by this method with those obtained by the Penrose & Quastel procedure and by titration with indophenol. Determinations by the three methods were therefore carried out on urines diluted with water to concentrations which were found to be convenient for our main investigation and to which 0·01 % of *p*-hydroxyphenylpyruvic acid was then added. It will be seen from Table 1 that the Briggs and the indophenol titration procedures were not a reliable index of the relative quantities of the two tautomers present. Whilst the Penrose & Quastel method showed a progressive conversion to the ketonic form which was found to be complete after 19 hr., the Briggs reaction, which on theoretical grounds should have indicated the enolic form, accounted during the first hour for almost the entire *p*-hydroxyphenylpyruvic acid added and fell only after 19 hr. to about half the original value.

Table 1. p-*Hydroxyphenylpyruvic acid found in diluted guinea-pig urine containing 0·01 % (w/v) of the compound*

(0·5 mg. present in each test)

Dilution	1/10			1/100		
Time after addition	By indophenol titration (mg.)	By Briggs test (mg.)	By Penrose & Quastel test (mg.)	By indophenol titration (mg.)	By Briggs test (mg.)	By Penrose & Quastel test (mg.)
0 min.	0·25	0·41	0·14	0·25	0·40	0·03
20 min.	0·24	0·47	0·19	0·25	0·44	0·05
40 min.	0·23	0·47	0·25	0·25	0·44	0·07
60 min.	0·21	0·44	0·31	0·25	0·44	0·10
19 hr.	0·01	0·22	0·50	0·05	0·25	0·38

Comparative experiments on the determination of *p*-hydroxyphenylpyruvic acid by the Briggs method in aqueous solution with and without the addition of salts have shown that the problem required a detailed investigation before the method could be used with any degree of reliance. The same applied to the indophenol titration procedure except, as already mentioned, in aqueous solutions in the absence of salts, when satisfactory results could be obtained in the assessment of the enolic form. We did not, however, investigate the matter further, as we had convinced ourselves that the Penrose & Quastel reaction was reliable enough for our purpose and supplied us with the necessary information.

The discrepancy between our results and those of Medes (1932), who was unable to isolate the phenylhydrazone, semicarbazone, or bisulphite compound from the urine of a patient excreting *p*-hydroxyphenylpyruvic acid cannot at present be explained.

SUMMARY

1. In aqueous solution the conversion of *p*-hydroxyphenylpyruvic acid from the enolic to the ketonic form was greatly accelerated in alkaline solution.

2. In guinea-pig urine, the conversion to the ketonic form took place more rapidly than in water at all values of pH investigated. Probably the conversion proceeds in guinea-pig tissue at a rate similar to that in urine. In both cases approximately 99 % of the *p*-hydroxyphenylpyruvic acid was found to exist in the ketonic form.

3. The determination of *p*-hydroxyphenylpyruvic acid in guinea-pig urine by the Briggs reaction or by indophenol titration does not yield satisfactory results, but the Penrose & Quastel reaction is reliable.

One of us (H.A.P.) is indebted to the Medical Research Council for a whole-time grant.

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The Ultraviolet Absorption Spectrum of Human Cerebrospinal Fluid

By M. H. HACK, *Department of Psychiatry, University of Illinois College of Medicine, Illinois Neuropsychiatric Institute, Chicago, Illinois*

(*Received* 21 *February* 1947)

Ultra-violet absorption spectra for specimens of human cerebrospinal fluid are illustrated in Fig. 1. The curves differ in shape and in the intensity and wave-length of maximum absorption. This suggests that there are at least two absorbing constituents. The data to be presented indicate that they are predominantly protein and ascorbic acid.

EXPERIMENTAL

All samples were portions of the whole of the clear and colourless cerebrospinal fluids (C.S.F.) withdrawn for pneumoencephalography, and the examination of each sample was completed within 30 min. The C.S.F. was obtained from an unselected group of patients (34 cases) from the neurological service. Our data are insufficient to

warrant any conclusion relating the spectrum to the pathologic condition, consequently this report is concerned only with the interpretation of the form of the spectrum.

The C.S.F. was diluted with equal parts either of 0·85% NaCl solution or of Sörensen phosphate buffer solution of pH 7·6 and compared with an appropriate blank in a Beckman quartz spectrophotometer at wave-lengths from 230 to 300 mμ. in steps of 2 mμ. The curves obtained by dilution of the C.S.F. with NaCl solution were identical with those diluted with phosphate buffer.

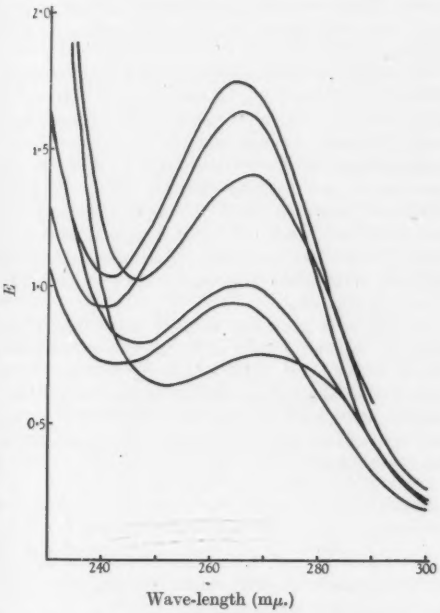

Fig. 1. Absorption spectra of C.S.F. from six patients. Each sample was diluted with an equal part of 0·85% sodium chloride solution.

RESULTS

The protein precipitated from 2 ml. of C.S.F. by 2 ml. of 10% trichloroacetic acid and dissolved in 4 ml. of saline showed maximum absorption at 280 mμ. (Fig. 2, *E*). These precipitates contained no phosphorus (Ma, Haas & Horecker, 1940) and consequently no nucleoprotein, nucleic acid or nucleotide. The globulin precipitated from 2 ml. of C.S.F. by 2 ml. of a saturated aqueous ammonium sulphate solution and dissolved in 4 ml. of saline, had the same spectrum except that all extinctions were somewhat less. The wave-length of the maximum of these spectra was independent of the pH, except when the solution was very alkaline. Then a maximum, attributable to tyrosine (Stenstrom & Reinhard, 1925), appeared at 290 mμ.

C.S.F. filtered at a pressure of 100–140 mm. Hg through Bechhold-Koenig porcelain crucibles (Alexander, 1926) impregnated with Parlodion (Mallinckrodt) and diluted with an equal part of phosphate

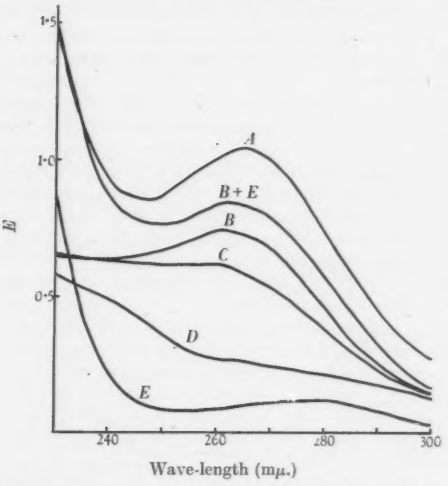

Fig. 2. Absorption spectrum: *A*, of C.S.F. diluted with an equal part of phosphate buffer solution of pH 7·6; *B*, of fresh ultrafiltrate (shown by titration to contain 1·3 mg. of ascorbic acid/100 ml.); *C*, of *D* to which ascorbic acid had been added to make a concentration of 1·1 mg./100 ml.; *D*, of ultrafiltrate *B* after exposure to room air for 120 min.; *E*, of trichloroacetic acid precipitate from 2 ml. of *A* dissolved in 4 ml. of 0·85% sodium chloride solution; *B* + *E*, arithmetic addition of *B* and *E*.

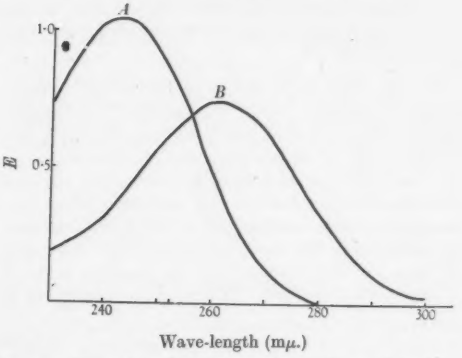

Fig. 3. Absorption spectra: *A*, of ascorbic acid in 0·2N-HCl; *B*, of ascorbic acid in phosphate buffer solution of pH 7·6.

buffer of pH 7·6 gave an absorption spectrum similar to that of ascorbic acid at the same pH (Figs. 2, *B* and 3, *B*). This spectrum disappeared com-

pletely, usually within 60 min., when the ultrafiltrate was exposed to air at room temperature (Fig. 2, *D*). When to such a preparation an appropriate quantity of ascorbic acid was added, its spectrum was restored to the form of the original ultrafiltrate (Fig. 2, *C*, *B*). The amount of ascorbic acid required was that determined by titration of the original ultrafiltrate with 2:6-dichlorophenol-indophenol.

The arithmetic addition of the protein spectrum (Fig. 2, *E*) and of the ultrafiltrate spectrum (Fig. 2, *B*) gives a curve (Fig. 2, *B*+*E*) which reasonably approximates the C.S.F. curve (Fig. 2, *A*) if allowance is made for the fact that the spectrophotometric examination requires time, during which the ascorbic acid is being oxidized and consequently disappears from the spectrum.

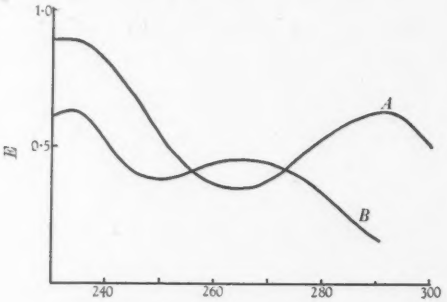

Fig. 4. *A*, absorption spectrum of an oxidized ultrafiltrate of C.S.F. (of *D*, Fig. 2) to which uric acid had been added to a final concentration of 0·5 mg./100 ml.; *B*, typical pyrimidine absorption spectrum obtained from 1·2 mg. aneurin hydrochloride in 100 ml. of a phosphate buffer solution of pH 7·6.

It has been reported (Spiegel-Adolf, Henny, Wycis & Spiegel, 1945; Spiegel-Adolf & Spiegel, 1945; Spiegel-Adolf, Wycis & Spiegel, 1946) that the normal C.S.F. of dogs show 'end absorption only', whereas the C.S.F. of dogs after electrically induced convulsions and of dogs, cats and men after concussion show spectra apparently identical with those reported here. The oxidation of ascorbic acid may account for the reported lack of maxima in C.S.F. of the control animals. Because the maximum at 265 mμ. reported in the experiments of Spiegel-Adolf *et al.* (1945, 1946) had been attributed to the purine-pyrimidine moiety of thymonucleic acid on the basis of traces of colour obtained by the diphenylamine method (Dische, 1930), the effect of ascorbic acid on this reaction was investigated. The addition of ascorbic acid in amounts similar to those found in C.S.F., to an extract of lymphatic tissue (cat) suppressed the reaction with the diphenylamine reagent, if the extract by itself gave only a trace of colour. Moreover, the production of colour in controls was prevented by such concentrations of ascorbic acid.

Uric acid in concentrations up to 1 mg./100 ml. is said to be a normal constituent of C.S.F. (Katzenelbogen, 1935). Such concentrations of uric acid readily passed through the ultrafilter and were demonstrated in the filtrate. Fig. 4, *A* shows the spectrum of a C.S.F. ultrafiltrate, minus the labile substance (ascorbic acid), to which sufficient uric acid had been added to give a concentration of 1 mg./100 ml. The resulting spectrum is that of uric acid and is different from any so far obtained from C.S.F. ultrafiltrates.

All patients received sodium pentothal intravenously not more than 15 min. prior to the withdrawal of the C.S.F. Since the barbiturates have an ultra-violet absorption spectrum (Stuckey, 1941) indistinguishable from that of uric acid, it is apparent that the sodium pentothal cannot be considered an interfering substance.

SUMMARY

1. Ascorbic acid and phosphorus-free protein preponderate in the absorption of ultra-violet light by human cerebrospinal fluid.

2. Ascorbic acid was shown to prevent the colour reaction of Dische's reagent with thymonucleic acid.

3. The presence of uric acid could not be demonstrated in the spectra of C.S.F. ultrafiltrates.

4. The use of intravenous sodium pentothal as an anaesthetic did not influence the spectrum of the C.S.F.

The author wishes to thank the members of the Department of Neurology and Neurosurgery for the prompt delivery of these cerebrospinal fluids.

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A Re-examination of Halibut-liver Oil. Relation between Biological Potency and Ultraviolet Absorption Due to Vitamin A

By R. A. MORTON and A. L. STUBBS (I.C.I. Fellow)
Biochemistry Department, University of Liverpool

(*Received* 3 *March* 1947)

Rather more than ten years ago, the Vitamin A Sub-Committee of the Accessory Food Factors Committee (Medical Research Council) organized large-scale co-operative biological assays on halibut-liver oil, with the object of ascertaining as accurately as possible the relationship between vitamin A potency and ultra-violet absorption at 328 mμ.

Two fairly large samples of oil were obtained and mixed in equal proportions. One sample was presented by Parke, Davis and Co., and the other, made by I. Spencer and Co. of Aberdeen, was obtained through the Torry Research Station, Aberdeen. The mixed oil was used by various groups of workers for feeding tests on properly prepared rats, using the responses to known dosages of the 1934 Standard β-carotene solution issued by the Director of Biological Standards (National Institute for Medical Research), as the fundamental criterion of vitamin A activity. The two oils (denoted hereunder as the P.D., and S. oils, respectively) were tested spectroscopically at Liverpool in 1936, and sealed samples in brown glass ampoules are still available and bear the following information on the labels:

Parke, Davis halibut-liver oil 24. iii. 36. M.R.C.		Spencer halibut-liver oil 23. iii. 36. M.R.C.	
$E^{1\%}_{1\,cm.}$	617 mμ. 230	$E^{1\%}_{1\,cm.}$	617 mμ. 143
	583 mμ. 121		583 mμ. 74·5
	328 mμ. 74·4		328 mμ. 47

617 and 583 mμ. refer to the $SbCl_3$ colour test and 328 mμ. to the ultra-violet absorption.

The biological data were gathered together and a statistical examination was made (Hume, 1937, 1939; Irwin, 1944). For the mixed oil, the estimated biological activity expressed in i.u./g. (1 i.u. equivalent to 0·6 μg. β-carotene) divided by the intensity of absorption at 328 mμ., expressed in terms of $E^{1\%}_{1\,cm.}$, was 1570. In other words, when $E^{1\%}_{1\,cm.}$ 328 mμ. equals 1, the potency is 1570 i.u./g.

At that time it was believed that if the absorption curve obtained for a solution of a rich oil was the same as that obtained for an equivalent solution of its unsaponifiable fraction there was no irrelevant absorption, i.e. the full intensity of absorption was due to the vitamin, It is now found that the belief is not necessarily, nor even usually, justified.

A later co-operative experiment under the same auspices as the previous one made use of a carefully prepared solution of vitamin A β-naphthoate. This substance was available in crystalline form and, apart from the presence of a small amount of vitamin A_2, the specimen used must have been nearly pure. Statistical study of the relationship between biological potency (assessed by comparison of responses with responses to Standard β-carotene) and ultra-violet absorption at 328 mμ. (corrected for any contribution traceable to the β-naphthyl moiety) led to a conversion factor of 1770 (Hume, 1943; Irwin, 1944). A similar assay with cod-liver oil, led to a conversion factor of 1820 (Hume, 1939; Irwin, 1944).

The difference between the conversion factors of 1570 and 1770 has since remained difficult to explain, and the assessment of absolute potency as distinct from relative potency has not been free from uncertainty. A re-examination of the two samples of halibut-liver oil in 1946 has gone far to improve the position.

METHODS

The oils were dissolved in purified *cyclo*hexane and their absorption curves determined using a Beckman photoelectric spectrophotometer. The $SbCl_3$ colour test (Carr-Price reagent) was carried out by measuring intensities of absorption at 617 and 583 mμ. using a Hilger-Nutting visual spectrophotometer. The following data were obtained:

		P.D. oil	S. oil
$E^{1\%}_{1\,cm.}$	617 mμ.	172	145
	583 mμ.	95	77
	328 mμ.	70·65	46·2 (ampoule *a*)
			47·2 (ampoule *b*)

By comparison with the 1936 tests the S. oil had kept very well, but the P.D. oil had undergone appreciable change.

It has been shown (Morton & Stubbs, 1946) that for vitamin A preparations free from detectable irrelevant absorption:

$$E\ 313\ m\mu. = E\ 338{\cdot}5\ m\mu.,$$

and

$$E\ 328\ m\mu./E\ 338{\cdot}5\ m\mu. = 1{\cdot}166.$$

The above criteria were satisfied by the purest preparations of crystalline vitamin A acetate dissolved in *cyclo*hexane. In the paper cited a simple method for estimating irrelevant absorption at 328 mμ. was proposed and tested. By

applying the method to the values of E at 313, 328 and 338·5 mμ. obtained for the two oils in *cyclo*hexane, the *corrected* intensities of absorption were

	P.D. oil	S. oil
$E^{1\%}_{1\,cm.}$ 328 mμ. (corr.)	46·9	40·2 (*a*) 40·0 (*b*)

Now the complete absorption curve for vitamin A over the wave-length range 250–370 mμ. is known with considerable accuracy (Morton & Stubbs, 1946) and the intensities of

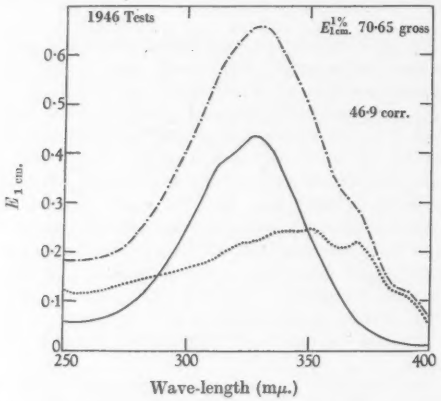

Fig. 1. Absorption spectrum of 'P.D.' halibut-liver oil 0·00932 % in *cyclo*hexane, 1 cm. cell. Sample stored in sealed ampoules 1936–46. —·—· Observed. —— Corrected curve. ····· Difference curve.

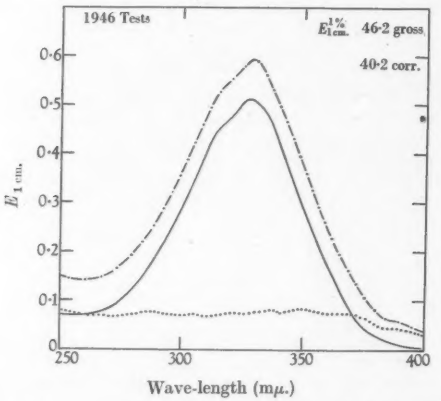

Fig. 2. Absorption spectrum of 'S.' halibut-liver oil 0·0127% in *cyclo*hexane, 1 cm. cell. Stored in sealed ampoules 1936–46. —·—· Observed. —— Corrected curve. ····· Difference curve.

absorption at various wave-lengths (5 mμ. intervals) have been recorded on a scale such that E 328 mμ. is 1·0. Given any value for E 328 mμ. (corr.) the complete corrected curve may at once be constructed. When this is done for the P.D. and S. oils (Figs. 1, 2) it becomes possible, by subtracting the corrected curve from the observed curve, to obtain the complete curve for irrelevant absorption. In so doing, the only assumption made is that between 313 and 338·5 mμ. this is sensibly linear.

RESULTS

Inspection of the subtraction curve in Fig. 1 (P.D. oil) reveals the presence of several maxima at positions coinciding with those already known to be characteristic of the so-called cyclized or anhydrovitamin A. The corresponding subtraction curve in Fig. 2 (S. oil) shows very much less intense irrelevant absorption, and the narrow maxima are only barely detectable. In the P.D. oil nearly one-third of the gross absorption at 328 mμ. is irrelevant in the sense that it is not due to vitamin A, whereas in the S. oil the correction is only about one eighth of the gross value. Since the latter oil shows no detectable change over the ten-year period of storage, it may be assumed that the irrelevant absorption has remained unchanged.

It is therefore justifiable to argue that a more satisfactory conversion factor for vitamin A can be obtained from the results of the original co-operative assay on halibut-liver oil by dividing the observed potency by the corrected value of $E^{1\%}_{1\,cm.}$ 328 mμ. Unfortunately, the known potency applies to a mixture of the two oils in equal proportions, and the amount of correction which should be applied to the original value of $E^{1\%}_{1\,cm.}$ 328 mμ. for the P.D. oil is not known. However, it seems that the two oils were originally very similar spectroscopically since there was very good agreement between the two samples for the ratios E 617 mμ./E 583 mμ. and E 617 mμ./E 328 mμ. as shown in Table 1. These ratios also provide further confirmation for the assumption that the S. oil has changed very little over the ten-year period of storage.

Table 1. *Relative intensities of absorption in the* $SbCl_3$ *colour test and at* 328 *mμ. for two samples of halibut-liver oil, in* 1936 *and* 1946

Oil	E 617 mμ./E 583 mμ.	E 617 mμ./E 328 mμ.
P.D. (1936)	1·90	3·09
S. (1936)	1·92	3·05
P.D. (1946)	1·81	2·43
S. (1946)	1·88	3·14

Since, then, the two oils were originally similar, the correction for irrelevant absorption which is now found necessary for the stable S. oil, should be applied to the original spectroscopic data on both oils. If this had been possible the conversion factor

as determined by the co-operative assay would have been $1570 \times 46{\cdot}7/40{\cdot}1 = 1828$. A value of 1830 for the conversion factor, obtained from assays on halibut-liver oil now falls into line with the value of 1770 obtained from the assays on crystalline vitamin A β-naphthoate, and 1820 obtained using cod-liver oil. In the latter case, the spectroscopic measurements were carried out on the non-saponifiable fraction of the oil, the irrelevant absorption in which is in general almost negligible (Morton & Stubbs, 1946).

The subtraction curves in Figs. 1 and 2, indicate the presence of anhydro-vitamin A ($\lambda_{max.}$ 351, 371 and 392 mμ.), with a suggestion of further selective absorption below 300 mμ. The validity of the subtraction procedure depends, however, upon a rather high degree of accuracy not only in the curves for the P.D. and S. halibut-liver oils, but also in the standard vitamin A curve, and furthermore the assumption of linearity in the irrelevant absorption between 313 and 338·5 mμ. is crucial. It would be more satisfactory, both as regards the accuracy of $E^{1\%}_{1\,cm.}$ 328 mμ. (corr.), and the characterization of the absorbing contaminants, if the latter could be separated from the vitamin A and the entire argument checked. The results of two different fractionation procedures are valuable in this respect.

The low temperature fractions (1–8) show little evidence of the presence of vitamin A, but their absorption spectra possess well-defined fine structure with the most prominent band at $\lambda_{max.}$ 367–370 mμ., indicating the presence of anhydro-vitamin A. These fractions also show selective absorption below 300 mμ. ($\lambda_{max.}$ 272 and 262 mμ.) in qualitative agreement with the subtraction curves. (The spectroscopic data obtained in 1939 on the original mixed oils suggest that the P.D. oil had probably started to deteriorate, since the absorption bands of anhydro-vitamin A appear as well-marked inflexions.)

(ii) The oils (1 g. of each) were saponified and the unsaponifiable matter extracted quantitatively with redistilled ether. The solvent was removed and the residue taken up in purified light petroleum. This solution was then extracted 12 times with aqueous ethanol (83 ml. absolute ethanol, 17 ml. water). The petroleum fraction was made up to 50 ml. and dried with anhydrous sodium sulphate. A portion was diluted with *cyclo*hexane for spectroscopic examination. The aqueous ethanol fraction was diluted with a large volume of water and exhaustively extracted with ether. After removal of the ether from the extract, the residue was transferred to *cyclo*hexane for spectroscopic examination. The results are shown in Table 3.

Table 2. *Molecular distillation of a mixture of two halibut-liver oils (P.D. and S.)*

Fraction	Temp. (°C.)	Wt. (g.)	Ultra-violet absorption maxima (mμ.) (a)	(b)	(c)	Intensities of absorption ($E^{1\%}_{1\,cm.}$) (a)	(b)	(c)
1	80	3·7	367	273	263	24	4·4	3·7
2	90	5·08	370	272	262	36·6	5·6	4·8
3	100	4·18	369	272	262	46·6	7	6
4	110	5·02	369	272	262	50	7·5	6·25
5	120	4·60	368	272	262	38	6·5	5
6	130	4·37	369·5	272	262	21·16	4·8	3·8
7	140	4·83	370	~272	—	9	3·0	—
8	150	4·32	~370	328	—	3	8·66	—
9	160	4·12	~389	328	—	1·4	11·25	—
10	170	4·66	—	328	—	—	23·5	—
11	180	4·28	—	328	—	—	57	—
12	190	3·54	—	328	—	—	109·3	—
13	200	3·22	—	328	—	—	166·7	—
14	210	3·42	—	328	—	—	224	—
15	220	3·80	—	328	—	—	205	—
16	230	3·73	—	328	—	—	158·7	—
17	240	5·88	—	328	—	—	86·6	—
18	250	6·28	—	328	—	—	37·5	—
Original oil			~391 ~368	328	—	14 29	64·5	—

(i) The mixed oils (91·9 g.) were mixed with 60 ml. of constant yield oil and the mixture subjected to molecular distillation by Dr T. H. Mead of British Drug Houses, Ltd. Table 2 shows the spectroscopic results obtained in 1939 on the resulting fractions.

It will be seen that the vitamin A is almost quantitatively extracted by the 83 % ethanol, giving an absorption curve requiring very little correction and agreeing very well with the corrected values of $E^{1\%}_{1\,cm.}$ 328 mμ. obtained for the original oils. The light

Table 3. *Partition of halibut-liver oil unsaponifiable fractions between light petroleum and 83 % aqueous ethanol*

	P.D. oil		S. oil	
	Petrol fraction	83 % ethanol fraction	Petrol fraction	83 % ethanol fraction
$E^{1\%}_{1\,cm.}$ 328 mμ. Gross (calc. on original oil)	16·8	48·25	2·9	39·4
$E^{1\%}$ 328 mμ. Corrected (calc. on original oil)	3·1*	45·5	0·6*	37·8

* Quantitative validity doubtful owing to questionable linearity of irrelevant absorption.

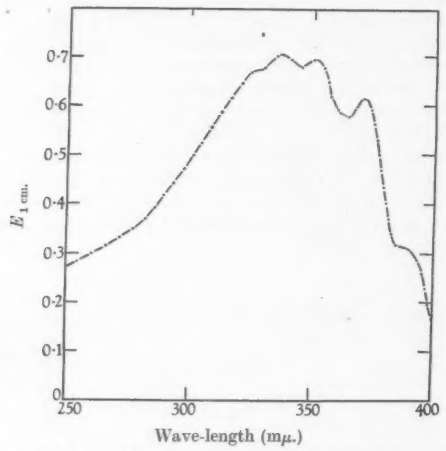

Fig. 3. Absorption spectrum of 'P.D.' halibut-liver oil; unsaponifiable fraction partitioned between 83 % ethanol and light petroleum; petroleum fraction 0·04 % in *cyclo*hexane, 1 cm. cell.

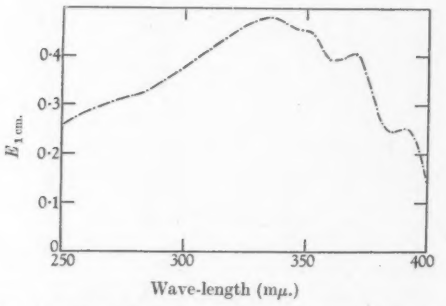

Fig. 4. Absorption spectrum of 'S.' halibut-liver oil; unsaponifiable fraction partitioned between 83 % ethanol and light petroleum; petroleum fraction 0·2 % in *cyclo*hexane, 1 cm. cell.

petroleum fraction showed the characteristic absorption of anhydro-vitamin A (Figs. 3, 4). The application of the correction procedure to these curves indicated the presence of only very small amounts of vitamin A.

DISCUSSION

The application of photoelectric spectrophotometry to the examination of the halibut-liver oils used in the large-scale co-operative assays of 1936 has shown that the original values of $E^{1\%}_{1\,cm.}$ 328 mμ., ascribed solely to the absorption of vitamin A, should have been subjected to some correction for absorption due to other constituents of the oils. When this irrelevant absorption is allowed for by measurements at three wave-lengths, the original estimate of 1570 for the conversion factor is raised to 1830, whilst the irrelevant absorption is shown, by subtraction of the vitamin A contribution from the observed curves, to be due mainly to anhydro-vitamin A. The validity of the correction procedure can be demonstrated by fractionation of the oils.

The principal significance of these results is that a single conversion factor can be applied to spectroscopic measurements on fish-liver oils, on unsaponifiable fractions of fish-liver oils and on crystalline vitamin A esters, provided that proper correction is made for irrelevant absorption. The potencies in i.u./g. so obtained will then be in accord with the biological estimates, provided that the biological assays are carried out under strictly comparable conditions. Thus, the conversion factor of 1800 suggested by the present work only applies to the particular conditions of diet, dosage level, etc. under which the three co-operative assays were carried out; changes in these conditions might well lead to a conversion factor appreciably different from 1800.

If, then, the expression of vitamin A potency in terms of i.u./g. is to persist, and general usage suggests that it should, a conversion factor of 1800 would seem to be desirable, on condition that the values of $E^{1\%}_{1\,cm.}$ 328 mμ. are fully corrected for irrelevant absorption. This is not likely to give rise to any serious discrepancies at the present time, when the more precise evaluation of $E^{1\%}_{1\,cm.}$ 328 mμ. due unequivocally to vitamin A is not generally applied, since $E^{1\%}_{1\,cm.}$ 328 mμ. for the majority of commercial fish-liver oils requires a correction of approximately 12 %. That is, the potency obtained by multiplying $E^{1\%}_{1\,cm.}$ 328 mμ. (gross) by the present conversion factor of 1600 is close to that obtained by multiplying $E^{1\%}_{1\,cm.}$ 328 mμ. (corrected) by 1800. Whilst this is true for the majority of fish-liver oils, the exceptions obviously cannot be ignored, so that a spectroscopic method of assay must eventually be universally adopted which makes proper allowance for irrelevant absorption. When such a method is generally applied, the actual value of the conversion

factor is not of fundamental importance, since the corrected value of $E^{1\%}_{1\,cm.}$ 328 mμ. is just as informative. The use of the 1800 factor would preserve continuity since it forms a link between the co-operative assays and the 1934 International Standard β-carotene.

SUMMARY

1. A re-examination of the two halibut-liver oils used in the co-operative assays of 1936 has been carried out by photo-electric spectrophotometry. The results show that the original spectroscopic data required some correction for absorption at 328 mμ. by constituents other than vitamin A.

2. When such a correction is applied, the conversion factor relating $E^{1\%}_{1\,cm.}$ 328 mμ. to potency in i.u./g. is raised from 1570 to 1830, which agrees well with the values of 1770 obtained for crystalline vitamin A β-naphthoate and 1820 for cod-liver oil, and justifies a round figure of 1800.

3. The results of fractionation experiments on the oils support the method of correction for irrelevant absorption.

4. The significance of the results with respect to the spectrophotometric assay of vitamin A is discussed.

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The Choice of a Chelating Agent for Inactivating Trace Metals

1. A SURVEY OF COMMERCIALLY AVAILABLE CHELATING AGENTS

By A. ALBERT and W. S. GLEDHILL
Department of Organic Chemistry, University of Sydney, Australia

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The important part played by various heavy metals in metabolic processes is well recognized. In studies of such processes, the removal of part or all of a particular metal from active participation in a biological system may be desired. Microchemical reagents, particularly the chelating agents evolved in the last 30 years (Morgan & Drew, 1920; Morgan & Burstall, 1936), seem likely to prove valuable tools for such purposes and some such applications have already been made.

The iron-deficient medium devised by Waring & Werkman (1942) is an interesting example. These authors saturated a synthetic bacteriological medium with oxine (8-hydroxyquinoline) and then extracted it with chloroform, thus removing heavy metals in the form of oxine complexes; traces of manganese, zinc and copper were then added. This produced a medium that was deficient in iron and which supported the growth of certain bacteria in proportion as iron was added.

It is not easy to make a good choice of micro-analytical reagents in biological work because there are practically no data available as to which metals these reagents will inactivate under physiological conditions. Little help can be obtained from the analytical literature which usually stipulates that the solutions to be tested must first be boiled, or treated with strong acid or alkali, or even that all organic matter should be removed.

The present studies are intended as a contribution towards exploring this gap in knowledge. Their aim is to discover which reagents give visible signs of reacting with a series of biologically important metals at 37° and pH 7·3. The investigation is confined to those substances believed to act through chelation and hence it excludes a few reagents of comparable delicacy such as hydrogen sulphide and some oxidizing agents. The results of the present work show that several reagents which are widely used in analytical practice to remove traces of metal from solution are ineffective at pH 7·3 and 37°. Other reagents are found to retain their efficacy, even at great dilution and to show more or less specificity for the various metals.

METHOD AND MATERIALS

The metals were used in the form of sulphates (acetates in the case of calcium and lead) of analytical reagent quality. One ml. of a 0·0004 M solution of the metal was mixed with 2 ml. of 0·1 M-borate buffer of pH 7·3. One ml. of 0·004 M-reagent was then added and the mixtures incubated in stoppered flasks at 37° for 24 hr. This made a final strength of 0·0001 M-metal and 0·001 M-chelating agent. Usually two molecules of chelating agent combine with one ion of metal so that there is a fivefold excess of chelating agent present in most cases. Although such a large excess may not have been necessary in every case, it was borne in mind that the

type of reaction being studied is a reversible one and that the law of mass action will come into play.

It was realized that 0·025M-borate buffer is near the acidic limit of its usefulness, but phosphate buffer of the same strength formed precipitates with the solutions of the metals and so was unsuitable. Using the borate buffer, the final pH varied between 6·5 and 7·3 in various cases (glass electrode measurements). That the borate buffer did not enter into the complexes was confirmed by omitting the buffer in replicate experiments which were carefully neutralized to pH 7; identical results were obtained.

The choice of 0·0001M-metal was suggested by the fact that this is the highest concentration at which two of the metals (iron and lead) are compatible with the buffer. All the tests were repeated at a tenfold greater dilution which seemed to be near the limits of visible response for even the most delicate reagents. Tables 1 and 2 record the precipitation or colour change observed. It may be assumed that the amount of precipitation or colour change provides an index of the extent of the chelation, although this may not be so in every case (see discussion).

The chelating reagents used were in the form supplied by British Drug Houses Ltd., with the following exceptions: cadion was presented by its discoverer, Dr F. P. Dwyer; the two xanthates were presented by Timbrol Ltd. (Sydney) and recrystallized just before use. The reagents were dissolved in water or in ethanol (7·5% or weaker) with the following exceptions: no. 3, 10% ethanol; no. 6, 25% ethanol; nos. 9, 10 and 23, 10% ethanol plus 1 equiv. of NaOH; and nos. 15 and 28, 1 equiv. of NaOH. Blanks without metals were run for each reagent.

RESULTS

The results, given in Tables 1 and 2, record the visible changes seen after mixing solutions of the metals and chelating agents at pH 7 (approx.), and maintaining the solutions for 24 hr. at 37°. The same results were obtained at room temperature (20°). Precipitation or colour changes were often not immediate, but had usually reached completion in 5 min. The reagents are arranged in alphabetical order using the more familiar synonyms.

Because of the great practical importance of being able to remove a complex from the site being studied, it was decided to distinguish between those chelating agents which formed precipitates (whether coloured or not) with metals and those which gave only a colour change. To differentiate these types, listed in Tables 1 and 2 respectively, the terms 'hydrophobic chelating agent' and 'hydrophilic chelating agent' are suggested. The connecting link between the two classes is provided by 1-nitroso-2-naphthol (no. 12) and nitroso-*R*-salt (no. 26), the latter being derived from the former by insertion of two water-attracting sulphonic acid groups. Even no. 26 gives precipitates if used with stronger solutions of metals than is done here. Dithizone (no. 7), which gives precipitates under the conditions used here, is employed in chloroform solution by analysts so that the complexes will stay in solution and can be estimated colorimetrically.

The results of these chelation tests carried out at pH 7 and 37° show some correspondences with, and some abrupt divergences from, those obtained in normal analytical practice. Results which correspond well are given by cupferron, cupron, dimethylglyoxime, dithizone (*v.s.*), 1-nitroso-2-naphthol, 2:2′-dipyridyl and *o*-phenanthroline (nos. 1, 2, 4, 7, 12, 25 and 27 respectively).

Some results in which gross lack of correlation is observed invite closer attention. For example, picrolonic acid (no. 15) precipitates calcium, cobalt, copper and lead under analytical conditions (where stronger solutions may be used), but is without visible effect in the present tests. Again, dithiol (no. 6) and mercaptobenzthiazole (no. 11) fail to react with cadmium in the present tests; likewise chromotropic acid (no. 24) and thioglycollic acid (no. 29) do not give their usual colours with iron. These divergences may be attributed to the neutral conditions of the present tests; for example, analysts use chromotropic acid in acid solution and thioglycollic acid in the presence of ammonia.

The most striking result to be seen in the tables is the complete absence of reaction of calcium or magnesium throughout the series. Oxine (no. 13) is known to precipitate both these metals under alkaline conditions. Calcium is known to be precipitated by stronger solutions of picrolonic acid (no. 15) and sodium dihydroxytartrate osazone (no. 21) than are used here. Magnesium is commonly detected with diphenylcarbazide (no. 5), 1-hydroxyanthraquinone (no. 8), magneson I and II (nos. 9 and 10), Titan-yellow (no. 22), cadion (no. 23) and quinalizarin (no. 28), but alkaline conditions appear to be used in each case. Even traces of the fine blue precipitates commonly given by nos. 9, 10 and 23 would be obvious if formed in the present tests. These differences can only be attributed to the conditions of temperature, pH and dilution under which chelation has been sought in the present studies. Their importance will be discussed later.

Finally, it is seen that few reagents show true specificity for a metal and that a very large number of reagents will chelate copper.

The following non-chelating precipitating agents for metals were found virtually ineffectual under the conditions of our tests even when 5 equivalents of ammonium thiocyanate were added: acridine, allyl iodide-hexamine, α- and β-naphthoquinolines (i.e. benzoquinolines) and pyridine. 5:5′-Dibromosalicil (Kuhn 3065), which might appear from its formula to be a chelating agent, was also ineffectual.

DISCUSSION

The wealth of data concerning the importance of trace metals in the economy of living cells has suggested biological experiments involving suboptimal concentrations of various metals. Sometimes this

Table 1. *Hydrophobic chelating agents: response to metals at* pH 7 *and* 37° *of chelating agents commonly used in analysis to precipitate metals*

No.	Chelating agent	Mol. wt.	Ca^{++}	Mg^{++}	Mn^{++}	Zn^{++}	Fe^{++}	Fe^{+++}	Cd^{++}	Co^{++}	Pb^{++}	Cu^{++}	Used in analysis to precipitate
1	Cupferron (ammonium nitroso-phenylhydroxylamine)	155	–	–	–	–	+	++	–	–	–	+	Fe, Cu
2	Cupron (benzoinoxime)	227	–	–	–	–	–	–	–	–	–	+++	Cu, Mo
3	*p*-Dimethylaminobenzalrhodanine	264	–	–	–	+	–	–	+	–	–	+++	Cu, Ag, Au
4	Dimethylglyoxime	116	–	–	–	–	–	–	–	–	–	–	Ni, Bi, Au, Pd
5	Diphenylcarbazide	242	–	–	–	++	–	–	–	–	–	++	Mg, Hg
6	Dithiol (4-methyl-1:2-dimercapto-benzene)	156	–	–	–	–	–	–	–	++	+	+++	Cd, Co, Pb, Cu, Sn
7	Dithizone (diphenylthiocarbazone)	256	–	–	–	++	–	–	+	++	+++	++	Zn, Cd, Co, Pb, Cu, Hg, Ag
8	1-Hydroxyanthraquinone	224	–	–	–	–	–	–	–	–	–	+++	Mg, Al
9	Magneson I (*p*-nitrobenzeneazo-resorcinol)	259	–	–	–	–	–	–	–	–	–	–	Mg
10	Magneson II (*p*-nitrobenzeneazo-α-naphthol)	293	–	–	–	–	–	–	–	–	–	–	Mg
11	Mercaptobenzthiazole	167	–	–	–	–	–	–	–	–	+	+	Cd, Pb, Cu, Tl
12	1-Nitroso-2-naphthol	173	–	–	+	+	+	+	–	+++	++	++	Fe, Co, Cu, Pd
13	Oxine (8-hydroxyquinoline)	145	–	–	++	++	+	+	++	++	++	++	Ca, Mg, Mn, Zn, Fe^{++}, Fe^{+++}, Cd, Co, Pb, Cu, Be, Al, Mo, U, V, W, Ti, Ni, Bi, Ga, In, Zr
14	Phenylthiohydantoic acid	210	–	–	–	+	–	–	+	++	+	–	Co, Ni
15	Picrolonic acid*	264	–	–	–	–	–	–	–	–	–	–	Ca, Co, Pb, Cu, Th
16	Potassium ethyl xanthate	160	–	–	–	–	–	–	+	+	++	++	Cu, Mo
17	Potassium *sec.*-butyl xanthate	188	–	–	–	–	–	–	+	+	+++	+++	Cu
18	Rubeanic acid (dithiooxamide)	120	–	–	–	+	–	–	+	+	+	+++	Co, Cu
19	Salicylaldoxime	137	–	–	–	–	–	+	–	–	+	+++	Cu, Pd
20	Sodium diethyldithiocarbamate	171	–	–	+	+++	+	++	+++	+++	+++	+++	Zn, Cu
21	Sodium dihydroxytartrate osazone	370	–	–	–	–	–	–	–	–	–	+	Ca
22	Titan-yellow (sodium 4:4′-di-(6-methyl-2-benzthiazolyl)-diazo-aminobenzene-2:2′-disulphonate)	680	–	–	–	–	–	–	–	–	–	+	Mg

Key: – No ppt.; + trace of ppt. with 0·0001 M-metal and 0·001 M-reagent; ++ marked ppt. with 0·0001 M-metal and 0·001 M-reagent; +++ as ++ but trace of ppt. also with 0·00001 M-metal and 0·0001 M-reagent.

* It is not known for certain whether the metallic complexes of this reagent involve chelation.

Table 2. *Hydrophilic chelating agents: response to metals at* pH 7 *and* 37° *of chelating agents commonly used in analysis to give colours with metals*

No.	Chelating agent	Mol. wt.	Ca^{++}	Mg^{++}	Mn^{++}	Zn^{++}	Fe^{++}	Fe^{+++}	Cd^{++}	Co^{++}	Pb^{++}	Cu^{++}	Used in analysis to give colours with
23	Cadion (*p*-nitrobenzenediazoaminoazobenzene)	346	–	–	–	–	–	–	+	–	–	–	Mg, Cd
24	Chromotropic acid (sodium 1:8-dihydroxynaphthalene-3:6-disulphonate)	364	–	–	+ + +	–	–	–	–	–	+ + +	+ + +	Fe, Cr, Ti
25	2:2′-Dipyridyl	156	–	–	–	–	+ + +	–	–	–	–	–	Fe++
26	Nitroso-*R*-salt (sodium 1-nitroso-2-naphthol-3:6-disulphonate)	377	–	–	–	–	+ + +	+ +	–	+ + +	–	+ +	Co
27	*o*-Phenanthroline	180	–	–	–	–	+ + +	–	–	–	–	–	Fe++
28	Quinalizarin (tetrahydroxyanthraquinone)	272	–	–	+ +	+	+	+ +	+	+ +	+ +	+	Mg, Be, Al, Ga
29	Thioglycollic acid	92	–	–	–	–	–	–	–	+ + +	–	–	Fe^{++}, Fe^{+++}
30	Thiourea	76	–	–	–	–	–	–	–	–	–	+	Bi

Key: – No colour; + trace of colour with 0·0001 M-metal and 0·001 M-reagent; + + marked colour with 0·0001 M-metal and 0·001 M-reagent; + + + as + + but trace of colour also with 0·00001 M-metal and 0·0001 M-reagent.

goal has been sought by the use of synthetic media in which chemicals of exceptional purity have been used. An alternative and less laborious approach is through the inactivation of trace metals which are naturally present, particularly in any way that permits of their removal through an induced change in solubility.

The use of chelating agents for this purpose is undergoing development. Some results of promise have already been obtained by Waring & Werkman (1942) whose iron-deficient medium was described in the introduction to this paper. No less significant was the attempted raising of oats by Piper (1942) in a copper-deficient medium obtained similarly by extraction with dithizone and chloroform. Mention must also be made of the use of thiourea and salicylaldoxime to decrease the copper available to the alga *Chlorella* and to some oxidases (Green, McCarthy & King, 1939); also an attempt to remove copper from spontaneous mammary cancer in mice (Boyland, 1946). Kindred topics are the inhibition of fungi by oxine and the reversal of this inhibition by zinc (Zentmyer, 1944), and an investigation of the connexion between chemistry and antibacterial action in a series of chelating agents (Albert, Rubbo, Goldacre & Balfour, 1947).

Although the literature of chelation is capably reviewed in various monographs (e.g. Mellan, 1941), the information most required by the biological worker is not available. First of all, the biologist wishes to know which of the reagents, that combine so readily with the various metals under the highly unphysiological conditions of an analytical procedure, will continue to do so under biological conditions. The data given in Tables 1 and 2 should go some way towards supplying this information. In particular, it is shown that many common chelating agents for calcium and magnesium do not react under these conditions. The standard conditions of the present tests (pH 7 and 37°) have, of course, special reference to mammalian physiology and the results will not necessarily apply in circumstances where the pH is widely different. However, the effects of temperature variations in the biological range will probably not be found very great.

The classification of these reagents into 'hydrophobic', i.e. those that give precipitates (Table 1) and 'hydrophilic', i.e. those that give soluble colour changes with metals (Table 2), should be of practical value because the complexes furnished by hydrophobic reagents are those most likely to be removable by extraction, e.g. with chloroform. Where hydrophilic reagents are concerned, only positive results have significance for it is quite possible for a reagent and a metal to chelate without visible colour change. This is the case with the suppression of calcium ions (in water) by calgon (hexasodium metaphosphate) and of magnesium ions by the Kom-

plexons of Schwarzenbach, Kampitsch & Steiner (1945). Again, because the division between hydrophobic and hydrophilic chelating agents is not a hard and fast one, it is possible (but unlikely) that a reagent in Table 1 which is producing precipitates with, say, four metals is forming a soluble complex with a fifth, without a colour change taking place.

Thus far the treatment has been only semi-quantitative. The questions now arise whether chelation goes on at dilutions below that at which precipitation can be recognized and to what extent the removal of a complex, e.g. by extraction, drives towards completion a reaction between a reagent and a metal for which it has little affinity. It is evident that these questions cannot fully be answered until 'stability constants' have been determined for each reagent with each metal. The stability constants are given by the expression:

$$K_s = \frac{[\text{metal complex}]}{[\text{free metal}] \times [\text{chelating agent}]}.$$

Possession of a list of such constants would facilitate the choice of the chelating agent most avid for any particular metal. Conversely it would indicate the most effective metal for reversing the antibiological effects of a chelating agent. Finally, if stability constants were available for the complexes of metals with amino-acids and other cell constituents, it would bring about a better understanding of the distribution of metals in the cell and the distribution of metals between the cell constituents and any chelating reagent which had been added for experimental purposes. The fact that chelating agents added to biological material will be competing, not necessarily successfully, with chelating agents naturally present, requires some emphasis. There is evidence that trace metals can be held at a biological surface oriented in such a way that only chelating agents of appropriate architecture can have access to them (Albert *et al.* 1947).

Unfortunately, the determination of the stability constants of chelate complexes presents formidable difficulties to the physical chemist. The small literature of this subject is concerned almost entirely with a search for practicable methods (Calvin & Wilson, 1945; Calvin & Bailes, 1946; Duffield & Calvin, 1946). With two exceptions the constants that have been obtained are without absolute significance in biology since they were determined in highly unphysiological solvents (pyridine, glacial acetic acid). The two exceptions are *o*-phenanthroline (no. 27) and dipyridyl (no. 25) which have K_s values of $10^{17.7}$ and 10^{15} (approx.) respectively for their complexes with ferrous iron in water (Dwyer & Nyholm, 1946), figures indicating that these particular reactions go a very long way towards completion even though no precipitation takes place. Already it is evident from Calvin's work that stability constants can vary between wide limits and that even in a series of closely related chelating agents some will hold a given metal tenaciously whereas others, because of steric or electronic influences in the molecule, can form appreciable amounts of complexes only in the presence of a considerable excess of metallic ions.

One of the most important conclusions to be drawn from Tables 1 and 2 is that too few reagents exist with specificity for particular metals. Modern knowledge of atomic radii and residual affinities can provide some of the data that the synthetic chemist requires to fill these gaps.

SUMMARY

1. Thirty organic reagents, in particular those used in analysis to combine with traces of metallic ions by means of chelation, have been examined at 37° and pH 7. The biologically interesting metals with which they were tested were calcium, magnesium, manganese, zinc, iron, cadmium, cobalt, lead and copper.

2. It is found that in many cases the combination between reagent and metal that occurs so readily under the unphysiological conditions of an analytical procedure does not take place under the standard conditions. In other cases the correspondence is good.

3. The usefulness of such tests as a guide to planning biological experiments aimed at removing selected trace elements is discussed critically.

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The Choice of a Chelating Agent for Inactivating Trace Metals

2. DERIVATIVES OF OXINE (8-HYDROXYQUINOLINE)

By A. ALBERT and D. MAGRATH
Department of Organic Chemistry, University of Sydney, Australia

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Oxine has been shown to retain its avidity for a number of biologically interesting metallic ions at pH 7 and 37° (Albert & Gledhill, 1947) and to possess a much more powerful antibacterial activity than other commercial chelating agents (Albert, Rubbo, Goldacre & Balfour, 1947). Hence it seemed desirable to investigate the isomerides and derivatives of oxine in order to correlate chelating activity, under physiological conditions of pH and temperature, with molecular structures. It seemed that such an elementary study might pave the way for the discovery of more delicate or selective reagents for depriving cells of essential trace metals. In addition, it was hoped to shed some light on the antibacterial action of oxine, which has been used as an antiseptic since 1895, mainly in the form of 'Chinosol' (Potassii Hydroxyquinolini Sulphas, B.P.C.), an acidic powder containing 50% oxine.

Forty-five analogues and derivatives of oxine have accordingly been investigated, with the result that the ability to chelate with a number of metals at pH 7 and 37° has been found to be widely distributed throughout the series. Certain of the derivatives gave visible evidence of activity at greater dilution than does oxine, but no outstanding instance of specificity was found. Some fundamental correlations between chemical constitution and chelating power and antibacterial activity have been brought to light.

METHOD AND MATERIALS

The tests were carried out exactly as in Part I (Albert & Gledhill, 1947). The derivatives and analogues of oxine were synthesized by the methods cited by references in Tables 1–3. The following details concern improved syntheses and new compounds.

4-Hydroxyquinoline. Aniline (10 ml.), aniline hydrochloride (0·1 g.) and freshly distilled ethyl oxalacetate (20 ml.) were mixed in a wide dish and kept *in vacuo* over $CaCl_2$ for 2 days. The product was diluted with ether (100 ml.) and extracted with 0·2N-HCl (two lots of 100 ml.), then with 0·2N-NaOH (three lots of 100 ml.), then with water (two lots of 50 ml.). The upper layer was dried over Na_2SO_4, then over K_2CO_3, filtered and completely freed from ether by vacuum distillation. The orange residue of diethyl anilinofumarate (20 g.) was poured into mechanically stirred medicinal paraffin (300 ml.) held at 250–255° (internal temp.) during 3 min., the flask being rinsed with a little light petroleum (b.p. 90–120°). The mixture was stirred for 10 min. more at 250–260° giving a golden liquid with white crusts. Light petroleum (60 ml.; b.p. 90–120°) was stirred in after cooling and the white crystals of ethyl-4-hydroxyquinoline-2-carboxylate were filtered off giving 13 g. (m.p. 210°) after washing with light petroleum.

This ester was heated with 2·5N-NaOH (200 ml.) at 97° for 2 hr. and the clear solution was acidified with HCl while hot, then cooled and filtered. This gave 11 g. of the acid as small, cream-coloured crystals, m.p. 280° (placed in bath at 260°). This acid and 0·01 g. of copper bronze were added to mechanically stirred paraffin at 260–265° (100 ml.) as quickly as effervescence permitted and stirred for 5 min. more. The mixture was cooled to 100° and light petroleum (25 ml.) added. After cooling to room temp., the mixture was filtered and the white 4-hydroxyquinoline (7·7 g.; m.p. 197°; 50% yield) washed with light petroleum and recrystallized from water (about 25 ml.) giving white crystals, m.p. 200°, not depressed by admixture with 4-hydroxyquinoline prepared by older procedures.

5-Propyloxine. *p*-Propylphenol, obtained in 80% yield by a Clemmensen reduction of *p*-hydroxypropiophenone, was nitrated according to the directions of Baranger (1931), the reaction being initiated by the addition of a crystal of $NaNO_2$. The 2-nitro-4-propylphenol (65% yield) was hydrogenated, using Raney nickel, in ethanol (10 parts) giving 2-amino-4-propylphenol, which was purified by recrystallizing the hydrochloride from HCl (yield of base: 60%, m.p. 139°). Nitrobenzenesulphonic acid mixture (3 g. of nitrobenzene and 13 g. of 17% oleum, heated at 100° until a drop gave only a faint turbidity on dilution) was added to 2 g. of 2-amino-4-propylphenol, followed by water (7·5 ml.) and glycerol (4 ml.). The mixture was refluxed for 8 hr. with continuous stirring, the internal temperature being adjusted to 135±5° by evaporation (or addition) of water. The mixture was diluted with water and fractionally precipitated with ammonia, rejecting the tars first formed. Eventually a pale solid (2 g.) was obtained; this was dissolved in N-HCl (15 ml.), boiled with charcoal, and the filtrate precipitated with an equal volume of 10N-HCl. This gave 1·12 g. of 5-propyloxine hydrochloride, m.p. 245–246°; the liberated base formed white crystals, m.p. 60–61°. The only previous synthesis involved the action of propionyl chloride on oxine followed by selective reduction of the keto group: the yield was very poor (Rosenmund & Karst, 1941).

7-Chlorooxine. Previous preparations (Claus & Giwartowsky, 1896; Howitz & Witte, 1905) gave products of m.p. 145 and 146°, lower than that now recorded (156°). 2-Chloro-6-nitrophenol ,m.p. 70–71° (10 g.) (Barton & Linnell, 1945), was hydrogenated in ethanol (125 ml.) at

atmospheric temperature and pressure over Raney nickel. The solution was filtered and taken to dryness under CO_2. The crude product was continuously extracted (Soxhlet) with light petroleum which deposited buff crystals of a new compound, *2-chloro-6-aminophenol* in 80% yield, m.p. 80°. Recrystallization from light petroleum (b.p. 30–60°) gave white crystals, m.p. 81°, which were much more soluble in hot water than in cold and soluble in acetone, ethanol and benzene without temperature gradient. (Found: C, 50·1; H, 4·2; N, 9·7. C_6H_6ONCl requires C, 50·2; H, 4·2; N, 9·8%.)

2-Amino-6-chlorophenol (3·5 g.), nitrobenzenesulphonic acid mixture (27 g.), water (14 ml.) and glycerol (7·5 ml.) were heated for 7 hr. as described for 5-propyloxine. The mixture was cooled, diluted and fractionally precipitated with ammonia. The 7-chlorooxine (3·6 g.; 80% yield, m.p. 150°) was purified from benzene until the m.p. (156°, i.e. 158° corr.) remained constant. The other properties agreed with those given by the early workers. (Found: C, 60·0; H, 3·4; N, 7·75. Calc. for C_9H_6ONCl: C, 60·2; H, 3·4; N, 7·8%.)

4-Aminooxine. 4-Hydroxyoxine (3 g.) was refluxed with phosphorus oxychloride (70 ml.) for 2 hr. and the excess reagent was distilled off *in vacuo*. The residue, added to a mixture of ammonia and ice, gave a yellow solid which was filtered off and dried *in vacuo*, giving 3 g. of crude 4-chlorooxine, m.p. 235–240°. This was heated with phenol (30 g.) for 2 hr. at 160° while a stream of NH_3 was bubbled through. Conc. HCl was then added and the phenol removed by steam distillation. The residual solution was concentrated until crystals of 4-aminooxine hydrochloride were deposited (2·2 g.; m.p. 344°). These were dissolved in N-NaOH, treated with charcoal, filtered and the filtrate acidified with acetic acid. After removing a dark impurity the filtrate was concentrated to a small bulk and cautiously treated with $NaHCO_3$ until precipitation was complete. *4-Aminooxine* was obtained (35% yield) as creamy-white needles from water, m.p. 208°. It is moderately soluble in water to a pale yellow solution and gives no marked colour changes with acid or alkali. (Found: C, 67·5; H, 5·1; N, 17·4. $C_9H_8ON_2$ requires C, 67·4; H, 5·0; N, 17·5%.) This substance does not diazotize in aqueous solution and hence the $-NH_2$ group is in the 4- and not in the 8-position.

5-Nitrosooxine. A solution of $NaNO_2$ (12·5 g.) in water (45 ml.) was slowly added to a well cooled, and mechanically stirred, solution of oxine (24 g.) in 10N-HCl (45 ml.). The mixture was allowed to stand overnight, diluted with water (50 ml.) and filtered. The precipitate of 5-nitrosooxine hydrochloride was washed with ice water and dried *in vacuo* (yield, 75%). The free base was obtained by dissolving the salt in N-Na_2CO_3 and adding acetic acid at 60° until precipitation was complete. The crystals were filtered off and dried *in vacuo*. 5-Nitrosooxine decomposes about 235° and has no definite m.p. Hence this batch was recrystallized from ethanol until material consuming the theoretical amount of hydrogen was obtained.

5-Aminooxine. The published syntheses having proved unsatisfactory, 5-nitrosooxine (5 g.) in acetone (100 ml.) was hydrogenated over Raney nickel at room temp. and pressure. The catalyst was filtered off by gravity under CO_2 and the filtrate treated with 10N-HCl (6·5 ml.). The orange crystals (5·3 g.; 80% yield) were filtered off and dissolved in a little hot 0·01N-HCl. The solution was treated with charcoal, filtered and diluted with an equal volume of 10N-HCl while hot. Orange crystals of 5-aminooxine dihydrochloride, obtained on refrigeration, were dried *in vacuo* over KOH (m.p. 294–295°, 60% yield). The hydrochloride is fairly stable, but the base is easily oxidized. (Found: C, 46·5; H, 4·35; N, 11·95; Cl, 30·5. Calc. for $C_9H_8ON_2$, 2HCl: C, 46·3; H, 4·3; N, 12·0; Cl, 30·4%.)

5:7-Dinitrooxine. As this substance decomposes rather than melts at 320°, it was submitted to analysis. (Found: N, 17·8. Calc. for $C_9H_5O_5N_3$: N, 17·9%.)

5:7-Diaminooxine. There is no satisfactory preparation of this substance in the literature. 5:7-Dinitrooxine (1·0 g.) was hydrogenated as 5-nitrosooxine (above). The orange precipitate of 5:7-diaminooxine dihydrochloride was purified by dissolving in water at 50°, adding one drop of 10N-HCl and diluting to double volume with acetone. The red needles of *5:7-diaminooxine monohydrochloride hemihydrate* (yield 60%), m.p. 275° (dec.), were dried *in vacuo* over KOH. (Found: C, 49·1; H, 5·0; N, 19·2; Cl, 15·9. $C_9H_9ON_3$, HCl, $0{\cdot}5H_2O$ requires C, 49·0; H, 5·0; N, 19·05; Cl, 16·1%.)

5-Diguanidinooxine. 5-Aminooxine dihydrochloride (2 g.), dicyandiamide (0·8 g.) and water (10 ml.) were heated at 97° for 2 hr. in CO_2. The solution was cooled, saturated with HCl and precipitated with acetone. The yellow solid was filtered off, dissolved in a little boiling N-HCl and poured into excess acetone which deposited yellow crystals of *5-diguanidinooxine dihydrochloride* which were dried *in vacuo* over KOH (65% yield), m.p. 262–263°. (Found: C, 41·3; H, 4·5; Cl, 22·2. $C_{11}H_{12}ON_6$, 2HCl requires C, 41·6; H, 4·45; Cl, 22·35%.)

7-Acetamido-8-acetoxyquinoline. This was obtained whenever an attempt was made to prepare 7-acetamidooxine by acetylating 7-aminooxine, whether by the use of acetic anhydride and sodium acetate in ether (Matsumura, 1927) or by boiling with one equivalent of acetic anhydride in benzene. *7-Acetamido-8-acetoxyquinoline* formed white crystals from benzene (m.p. 172°). (Found: C, 63·5; H, 4·9; N, 11·4. $C_{13}H_{12}O_3N_2$ requires C, 63·9; H, 4·95; N, 11·5%.) An attempt to hydrolyze the *O*-acetyl group with 0·5N-NaOH gave a mixture of diacetylated and unacetylated material.

Ethyl ester of oxine-5-carboxylic acid. As the method of von Niementowski & Sucharda (1916) gave only a 30% yield, oxine-5-carboxylic acid (1 g.), H_2SO_4 (1 ml.) and absolute ethanol (20 ml.) were refluxed for 8 hr. The solution was concentrated to 7 ml., poured into water, made faintly alkaline with Na_2CO_3 and filtered. The precipitate was taken up in 40% aqueous ethanol, treated with charcoal, filtered and boiled until crystals began to form. The mixture was cooled in ice, filtered and the crystals extracted with hot benzene, giving white needles; m.p. 125°, in 70% yield.

5-Hydroxybenzo-f-quinoline (*5:6-benzooxine*, Fig. 2*c*). 2-Amino-3-methoxynaphthalene (3 g.), nitrobenzenesulphonic acid mixture (17·5 g.), water (8·5 ml.) and glycerol (5 ml.) were heated for 6 hr. as described under 5-propyloxine. Aqueous ammonia was then added and the plastic precipitate was dried and refluxed with HBr (30 ml.; sp.gr. 1·5) for 10 hr. The HBr was recovered and the residue diluted with water. Fractional precipitation with ammonia gave tar (discarded), followed by orange plastic material which became solid on drying and was extracted with N-NaOH. The filtrate was precipitated with acetic acid and the pale 5:6-benzooxine recrystallized from light petroleum (b.p. 60–90°). The yield was 6% of ivory-coloured crystals, m.p. 106–107°. The use of arsenic acid as oxidizing agent gave lower yields. (Found: C, 79·9; H, 4·6

Calc. for $C_{13}H_9ON$: C, 79·95, H, 4·65%.) Barnum & Hamilton (1942), who prepared this substance from 2-amino-3-naphthol in poor yield, give a lower m.p. (104–106°).

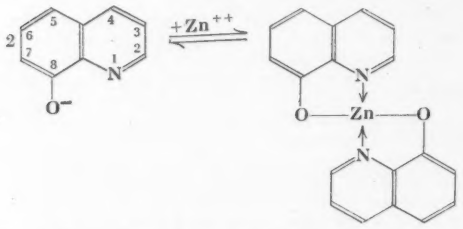

Fig. 1. Reaction between two oxine anions and one zinc ion to give oxine metal chelate complex.

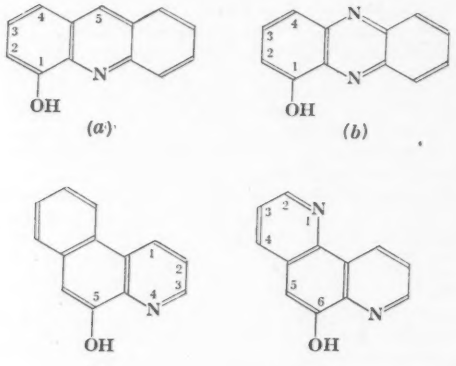

Fig. 2. (*a*) 1-Hydroxyacridine. (*b*) 1-Hydroxyphenazine. (*c*) 5-Hydroxy-benzo-*f*-quinoline (5:6-benzooxine). (*d*) 6-Hydroxy-*m*-phenanthroline.

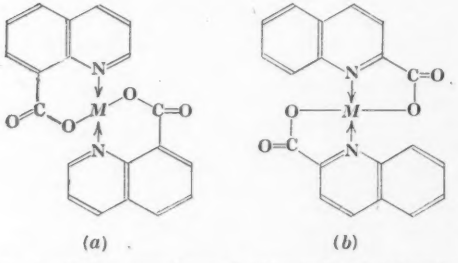

Fig. 3. Chelate complexes of (*a*) quinoline-8-carboxylic acid and (*b*) quinoline-2-carboxylic acid (quinaldinic acid) with a divalent metal (*M*).

RESULTS

The results of these tests are given in Tables 1–5 which record, semi-quantitatively, the precipitation of a number of metallic ions of biological interest by oxine and 45 substances related to it. This precipitation is caused by chelation involving the combination of one polyvalent metallic cation with as many anions of oxine as the cation has positive charges. For example, one zinc cation unites with two anions of oxine as in Fig. 1 (Fleck & Ward, 1933) and the combination involves new bonds, from nitrogen to metal and from metal to oxygen, (Morgan & Burstall, 1936).

When a metal combines with oxine the only water-attracting groups present (—OH and =N—) become masked, resulting in a strongly hydrophobic substance which is forced out of solution. Hence, among closely related substances, the amount of precipitation is likely to be roughly proportional to the extent of the chelation; conversely, the absence of precipitation usually implies that chelation has not occurred. Only when, in addition to the chelating groups, a strongly hydrophilic group (in particular —SO_3H or —COOH) is introduced, can the absence of precipitation be consistent with chelation having occurred in the concentrations dealt with here. When precipitation did not occur another test for chelation was applied, viz. an investigation as to whether hydrogen ions were liberated in the clear solution formed when solutions of the reagent and the salt of a divalent metal were mixed. This effect is exemplified in the following equation:

$$CuCl_2 + 2\overset{\text{Cu}}{\overset{\diagup\ \diagdown}{N}}\!-\!X\!-\!OH = 2\overset{\text{Cu}}{\overset{\diagup\ \diagdown}{N}}\!-\!X\!-\!O + 2H^+ + 2Cl^-$$

where *N*—*X*—OH stands for the derivative of oxine. Actually this liberation of hydrogen ions increases when alkali is added to the system so that, in the presence of the metal, the normal titration curve of the compound is very considerably displaced.

In Part I (Albert & Gledhill, 1947) this type of chelate activity was designated 'hydrophilic chelation' to distinguish it from the more common 'hydrophobic chelation' and is exemplified by oxine-5-sulphonic acid (Table 2, no. 31) which does not give precipitates with metals. The chelating tendencies of this substance, although not necessarily equal to those of oxine, are clearly revealed by the abnormal acid-base titration curve obtained in the presence of copper, as in Fig. 4. Quinoline-5-sulphonic acid, which cannot chelate, did not behave in this way.

Table 1 summarizes the interaction between the inorganic ions dealt with in Part I (Albert & Gledhill, 1947) and the seven isomeric monohydroxyquinolines. Quinoline is included for purposes of comparison. It can be seen that oxine alone gives precipitates with the metals. Further tests showed that copper salts did not liberate hydrogen ions from the isomerides of oxine. Hence it is established that oxine alone of its isomerides is capable of chelation.

Table 2 deals similarly with simple derivatives of oxine. A variety of substituents, both lipophilic and hydrophilic, electron-attracting and electron-repelling, acid-strengthening and acid-weakening, have been inserted in various positions in the oxine molecule. It can be seen that a high degree of chelation persists in spite of considerable variation in the type of substituent. Even in the cases of nos. 26, 29, 31 and 32 which give little or no precipitation, evidence of hydrophilic chelating powers was obtained from the copper test (*v.s.*). However,

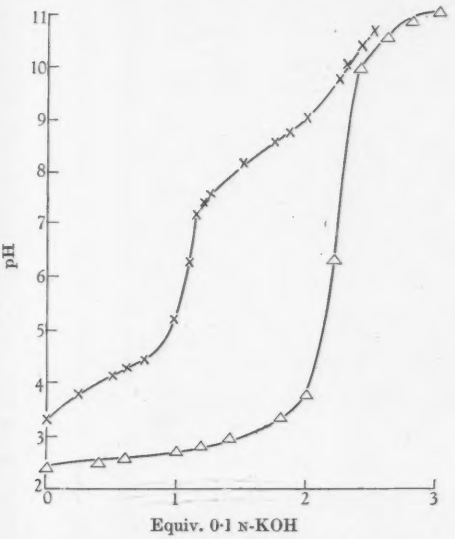

Fig. 4. Evidence of chelation from liberation of hydrogen ions (20° C.). × M/320 Oxine-5-sulphonic acid (pH 3·3). △ M/320 Oxine-5-sulphonic acid (pH 3·3) + M/640 cupric sulphate (pH 5·7).

it was found that the blocking of either the oxygen or the nitrogen atom (as in nos. 9, 10 and 12) completely prevented any union between the substance and the metal. A few of the compounds (nos. 22–28, inclusive) were found to undergo aerial oxidation catalyzed by the metal present. For example, 1:2:3:4-tetrahydrooxine (no. 28), in the presence of air, gave red solutions with Mn, Zn, Cd, Co, Pb and Cu instead of the stable yellow precipitates given by oxine and many of its derivatives.

Table 3 records the chelating properties of some substances having structural features in common with oxine. Four of these compounds belong to different heterocyclic series but possess the configuration of chelating atoms found in oxine (1-hydroxyacridine, 1-hydroxyphenazine, 5-hydroxybenzo-*f*-quinoline and 6-hydroxy-*m*-phenanthroline,

Table 1. *Oxine and isomerides: visible response to metals at* pH *7 and 37° with reagent in excess*

No.	Substance	Source	Criterion of purity	Mol. wt.	Ca^{++}	Mg^{++}	Mn^{++}	Zn^{++}	Fe^{++}	Fe^{+++}	Cd^{++}	Co^{++}	Pb^{++}	Cu^{++}
1	8-Hydroxyquinoline (oxine)	B.D.H., recryst. from dilute ethanol and from light petroleum	m.p. 75°	145	–	–	+ +	+ +	+	+	+ +	+ +	+ +	+ +
2	7-Hydroxyquinoline	Claus & Massau (1892)	m.p. 235°	145	–	–	–	–	–	–	–	–	–	–
3	6-Hydroxyquinoline	Skraup (1882)	m.p. 193°	145	–	–	–	–	–	–	–	–	–	–
4	5-Hydroxyquinoline	Claus & Howitz (1893)	m.p. 223°	145	–	–	–	–	–	–	–	–	–	–
5	4-Hydroxyquinoline	For synthesis, see text	m.p. 200°	145	–	–	–	–	–	–	–	–	–	–
6	3-Hydroxyquinoline	Mills & Watson (1910)	m.p. 198°	145	–	–	–	–	–	–	–	–	–	–
7	2-Hydroxyquinoline*	Einhorn & Lauch (1886)	m.p. 199°	145	–	–	–	–	–	–	–	–	–	–
8	Quinoline	B.D.H., refractionated in hydrogen	b.p. 238°	129	–	–	–	–	–	–	–	–	–	–

Key: – No ppt.; + trace of ppt. with 0·0001 M-metal and 0·001 M-reagent; + + marked ppt. with 0·0001 M-metal and 0·001 M-reagent; + + + as + + but trace of ppt. also with 0·00001 M-metal and 0·0001 M-reagent.

* No precipitate even with 0·005 M-reagent and 0·0005 M-Ca, Mg, Mn, Zn, Cd, Co, Cu (Fe and Pb were not tested at this strength as they are precipitated by the buffer).

Table 2. *Substituted oxines: visible response to metals at* pH 7 *and* 37° *with reagent in excess*

No.	Substance	Source	Criterion of purity	Mol. wt.	Ca^{++}	Mg^{++}	Mn^{++}	Zn^{++}	Fe^{++}	Fe^{+++}	Cd^{++}	Co^{++}	Pb^{++}	Cu^{++}
9	*O*-Methyloxine (8-methoxyquinoline)*	Bedall & Fischer (1881)	m.p. 46°	159	–	–	–	–	–	–	–	–	–	–
10	Oxine methochloride (8-hydroxy-*N*-methylquinolinium chloride)*	Claus & Howitz (1890)	m.p. 232° (dec.)	196	–	–	–	–	–	–	–	–	–	–
11	2-Methyloxine	Doebner & Miller (1884)	m.p. 74°	159	–	–	+ + +	+ + +	+	+	+ + +	+ + +	+ + +	+ + +
12	2-Methyl-8-methoxyquinoline	Doebner & Miller (1884)	m.p. 125°	173	–	–	–	–	–	–	–	–	–	–
13	5-Methyloxine	Nölting & Trautmann (1890)	m.p. 122–123°	159	–	–	+ +	+ + +	+ +	+ +	+ +	+ +	+ +	+ + +
14	5-*n*-Propyloxine	For synthesis, see text	m.p. 60–61°	187	–	–	+ +	+ +	+ +	+ +	+ +	+ +	+ + +	+ + +
15	5-Chlorooxine	D.R.P. (1930)	m.p. 125°	179·5	–	–	+ +	+ +	+	+	+ +	+ + +	+ +	+ + +
16	7-Chlorooxine	For synthesis, see text	m.p. 156°	179·5	–	–	+ +‡	+ +‡	+ +	+ + +	+ +	+ + +	+ +	+ + +
17	5:7-Dichlorooxine	D.R.P. (1930)	m.p. 181°	214	–	–	+‡	+ +	+ +	+ +	+ +	+ +	+ +	+ +
18	5-Chloro-7-iodooxine (Vioform)	CIBA, recryst.	m.p. 178–179°	305·5	–	–	+‡	+‡	+ +	+ +	+ +	+ +	+ +	+ +
19	5-Nitrooxine	Kostanecki (1891)	m.p. 178°	190	–	–	–	+	+ +	+ +	–	+ + +	+ +	+ + +
20	5:7-Dinitrooxine	Bennett & Grove (1945)	m.p. 320° (dec.), see also text	235	–	–	+	–	+	+	+	+	+	+
21	5-Nitrosooxine	For synthesis, see text	See text	174	–	–	–	+	(+)†	–	+	+ +	+	+ + +
22	4-Aminooxine§	For synthesis, see text	m.p. 208°	160	–	–	–	–	+	+	–	–	–	+
23	5-Aminooxine§	For synthesis, see text	See text	160										
24	7-Aminooxine§	Matsumura (1927), but hydrolysis conducted at 190° instead of 170°	m.p. 131°	160					Difficult to assess because of oxidation					
25	5:7-Diaminooxine§	For synthesis, see text	See text	175	–	–	–	–	+	–	–	–	–	–
26	5-Diguanidinooxine§	For synthesis, see text	See text	244	–	–	–	–	(+)†	(+)†	–	(+)†	–	–
27	4-Hydroxyoxine (8-hydroxy-4-keto-1:4-dihydroquinoline)§	Musajo & Minchilli (1941)	m.p. 314–315°	161	–	–	–	–	(+ +)†	–	–	(+)†	–	–
28	1:2:3:4-Tetrahydrooxine§	Bedall & Fischer (1881)	m.p. 121–122°	149	–	–	–	–	(+)†	(+)†	–	(+)†	–	–
29	Oxine-5-carboxylic acid*	Lippmann & Fleissner (1886)	m.p. 273° (dec.)	189	–	–	(+)†	–	(+)†	(+)†	–	(+)†	–	–
30	Ethyl ester of no. 29	Niementowski & Sucharda (1916)	m.p. 125–126°	217	–	–	+	+	+	+	+	+	+	+
31	Oxine-5-sulphonic acid*	Claus & Posselt (1890)	m.p. 321–322°	225	–	–	–	–	(+)†	(+)†	–	–	–	–
32	7-Iodooxine-5-sulphonic acid (Chiniofon; Ferron; Yatren)*	B.D.H., recryst.	m.p. 285° (dec.)	351	–	–	–	–	(+ +)†	(+)†	–	–	–	–

Key: – No ppt.; + trace of ppt. with 0·0001 M-metal and 0·001 M-reagent; + + marked ppt. with 0·0001 M-metal and 0·001 M-reagent; + + + as + + but trace of ppt. also with 0·00001 M-metal and 0·0001 M-reagent.

* No precipitate even with 0·005 M-reagent and 0·0005 M-Ca, Mg, Mn, Zn, Cd, Co, Cu (Fe and Pb were not tested at this strength as they are precipitated by the buffer).

† Colour change, no precipitate.

‡ Exact reading difficult as reagent is poorly soluble and controls become cloudy.

§ Metals appear to catalyze oxidation of these compounds causing colour changes.

§ Metals appear to catalyze oxidation of these compounds causing colour changes.

Table 3. *Substances chemically related to oxine: visible response to metals at* pH 7 *and* 37° *with reagent in excess*

No.	Substance	Source	Criterion of purity	Mol. wt.	Ca^{++}	Mg^{++}	Mn^{++}	Zn^{++}	Fe^{++}	Fe^{+++}	Cd^{++}	Co^{++}	Pb^{++}	Cu^{++}
			(*a*) Same chelation mechanism as in oxine, but different nucleus											
33	1-Hydroxyacridine (Fig. 2*a*)	Jensen & Rethwisch (1928)	m.p. 116°	195	–	–	++	++	+	++	++	+	++	+++
34	1-Methoxyacridine	Jensen & Rethwisch (1928)	m.p. 134°	209	–	–	–	–	–	–	–	–	–	–
35	2-Hydroxyacridine	Albert & Ritchie (1943)	m.p. 285°	195	–	–	–	–	–	–	–	–	–	–
36	1-Hydroxyphenazine (Fig. 2*b*)	Wrede & Strack (1929)	m.p. 157°	196	–	–	+++	+	–	–	+	+++	+++	+
37	1-Methoxyphenazine	Wrede & Strack (1929)	m.p. 169°	210	–	–	–	–	–	–	–	–	–	–
38	2-Hydroxyphenazine	Kehrmann (1924)	m.p. 253°	196	–	–	–	–	–	–	–	–	–	–
39	5-Hydroxybenzo-*f*-quinoline (5:6-benzoxine; Fig. 2*c*)	For synthesis, see text	m.p. 106–107°	195	–	–	+++	+++	++	++	+++	+++	+++	+++
40	6-Hydroxy-*m*-phenanthroline (5:6-pyridoxine; Fig. 2*d*)	Haworth & Sykes (1944)	m.p. 160°	196	–	–	+++	+*	++	++	+++	++	++	++
			(*b*) Same nucleus (quinoline) as in oxine, but different chelating mechanism											
41	8-Mercaptoquinoline	Edinger (1908)	m.p. 58–59°	161	–	–	+	+	+	+	+	+++	+	+++
42	8-Aminoquinoline	Hydrogenation of 8-nitroquinoline (Raney nickel)	m.p. 65°	144	–	–	–	–	–	–	–	–	–	–
43	Quinoline-2-carboxylic acid (quinaldinic acid; Fig. 3*b*)	B.D.H., recryst.	m.p. 157°	173	–	–	–	+	+	–	+	–	+	+
44	Quinoline-4-carboxylic acid (cinchoninic acid)	Pfitzinger (1902)	m.p. 253–254°	173	–	–	–	–	–	–	–	–	–	–
45	Quinoline-8-carboxylic acid (Fig. 3*a*)	Schlosser & Skraup (1881)	m.p. 187°	173	–	–	–	–	–	–	–	–	+	+++
46	4:8-Dihydroxyquinoline-2-carboxylic acid (xanthurenic acid)†	Musajo & Minchilli (1941)	m.p. 284–285°	205	Difficult to assess because of oxidation									

Key: – No ppt.; + trace of ppt. with 0·0001 M-metal and 0·001 M-reagent; ++ marked ppt. with 0·0001 M-metal and 0·001 M-reagent; +++ as ++ but trace of ppt. also with 0·00001 M-metal and 0·0001 M-reagent.

* Exact reading difficult as reagent is poorly soluble and controls become cloudy.

† Metals appear to catalyze oxidation of this compound causing colour changes.

nos. 33, 36, 39 and 40, see Fig. 2). It is evident that these substances have chelating powers very similar to those of oxine. Further, it is seen that, as with oxine, chelating ability is lost when the hydroxyl group is blocked (nos. 34 and 37) or else removed to a distant position (nos. 35 and 38). Table 3 also lists some quinoline compounds which differ from oxine in the chemical nature of the groups available for chelation. 8-Mercaptoquinoline (no. 41), in which the O of oxine has been replaced by S, retains chelating power, but 8-aminoquinoline (no. 42) shows no chelating properties under the conditions of these tests as its complexes are weakly held, water-soluble cations (Burrows & Ritchie, 1939). As chelation does not usually take place unless a 5- or 6-membered ring can be formed, it is understandable that 2-hydroxyquinoline (no. 7) does not chelate. Among the quinolinecarboxylic acids (nos. 43–45) both the 2- and 8-isomerides could reasonably be expected to chelate as in Fig. 3. Actually these substances chelate less avidly than oxine, quinoline-8-carboxylic acid (no. 45) being almost a specific reagent for Cu.

The results of Tables 1–3 were extended by the examination of some other cations of possible biological interest (Al, Ni, MoO_2 and UO_2). The results (Table 4) show selectivity, but confirm the impression that oxines are capable of chelation with many different metals, even under the restricted conditions of these tests.

equal to the maximal amount bound by the metal present in the earlier tests. There is substantial agreement between the results of the two tests.

All the tests described so far have been carried out at pH 7·3 and it was thought desirable to examine oxine under more alkaline conditions (pH 8·6). The results differed from those of Tables 1 and 4 in only one detail, viz. Mg was now precipitated.

At the request of our biological collaborators, we examined the effect of oxine on a series of complex cobaltic salts (conditions as in Table 1): sodium cobaltinitrite, chloropentamminocobaltic chloride, nitratopentamminocobaltic nitrate, dinitrotetramminocobaltic chloride, carbonatotetramminocobaltic nitrate and *trans*dichlorodiethylenediamine cobaltic chloride. In no case was there any reaction with oxine. However, freshly prepared cobaltic sulphate reacted similarly to any cobaltous salt.

To provide data for discussion of the effect of ionization upon chelation, the pK_a values (i.e. the negative logarithms of the acidic ionization constants) of oxine and 19 related substances were obtained and the percentages of the four species (anion, cation, zwitterion and neutral molecule) calculated for pH 7·3. It can be seen from Table 6 that the proportions of the four species vary greatly from compound to compound. Oxine (no. 1) is present principally as neutral molecule with only 0·5 % of cation and 0·7 % of anion, at 20°, the temperature of

Table 4. *Other metals: visible response to some other metals of possible biological interest at* pH 7 *and* 37°, *reagent in excess*

No.	Substance	Al^{+++}	Ni^{++}	MoO_2^{++}	UO_2^{++}
1	Oxine	+ +	+	+	+ +
15	5-Chlorooxine	+ + +	+ + +	+	+ + +
19	5-Nitrooxine	+ + +	–	–	–
21	5-Nitrosooxine	–	(+)*	–	–
40	6-Hydroxy-*m*-phenanthroline	+	+ + +	+†	+ + +

Key: Signs have same meaning as in Tables 1–3 (inclusive).

* Colour change, no precipitate.

† Exact reading difficult as reagent is poorly soluble and controls are cloudy.

Table 5. *Metals in excess: visible response of oxine to metals at* pH 7 *and* 37° *with metals in excess*

Ca^{++}	Mg^{++}	Al^{+++}	Mn^{++}	Zn^{++}	Fe^{++}	Fe^{+++}	Cd^{++}	Co^{++}	Ni^{++}	Pb^{++}	Cu^{++}	MoO_2
–	–	+	+	+ +	+ +	+ +*	+ + +	+ + +	+ +	+*	+ +	–

Key: – No ppt.
+ Trace of ppt. with 0·0004 M-metal and 0·0002 M-oxine.
+ + Marked ppt. with 0·0004 M-metal and 0·0002 M-oxine.
+ + + As + + but trace of ppt. also with 0·00004 M-metal and 0·00002 M-oxine.

* Exact reading difficult as control is cloudy.

As preliminary work indicated that oxine-metal reactions obeyed the mass action law, an excess of organic reagents had been used in all the tests described so far. It is interesting now to record the results (Table 5) of using the metals in excess. The quantity of oxine used in such tests has been made

the determinations. At 37° the proportions undergo only a small change (0·3 % cation and 1·5 % anion).

Surveying the table, it will be seen that the percentage of anion varies from $<0·01$ % (no. 26) to 83 % (no. 18) or even, if non-phenolic anions are considered, 98 % (no. 29). Likewise the percentage

Table 6. *Ionization of oxine and related compounds at 20°*

No.	Substance	pK_a		Reference	Percentage of species present at pH 7·3 in water: Anion	Kation	Zwitterion	Neutral molecule
1	Oxine	—OH	9·45	(1)	0·7	0·5	Nil	98·8
		=N—	5·03		(1·5 at 37°)	(0·3 at 37°)		
8	Quinoline	=N—	4·94	Albert & Goldacre (1944)	Nil	0·4	Nil	99·6
14	5-*n*-Propyloxine	—OH	(9·7)	(1), (2)	0·4	0·5	Nil	99·1
		=N—	(5·0)					
15	5-Chlorooxine	—OH	(8·7)	(1), (2)	4·3	<0·1	Nil	95·7
		=N—	(3·8)					
16	7-Chlorooxine	—OH	7·7	(1), (3)	28·5	<0·1	Nil	71·5
		=N—	(4·0)	(1), (2)				
17	5:7-Dichlorooxine	—OH	(6·7)	(1), (2)	80·0	<0·1	Nil	20·0
		=N—	(*c.* 3·6)					
18	5-Chloro-7-iodooxine	—OH	(6·6)	(1), (2)	83·4	—	Nil	16·6
22	4-Aminooxine	—OH	6·91	(1)	0·03	28·5	71·5	Nil
		=N—	10·71					
23	5-Aminooxine	—OH	11·24	(1)	<0·1	2·5	Nil	97·5
		=N—	5·67					
25	5:7-Aminooxine	—OH	6·96	(1)	0·02	28·9	71·1	Nil
		=N—	10·83					
26	5-Diguanidinooxine	—OH	8·89	(1)	<0·01	97·9 (<0·1 (=N⁺—))	2·1	Nil
		—C(=NH)NH$_2$	11·8					
		=N—	3·97					
28	1:2:3:4-Tetrahydrooxine	—OH	(9·4)	(1), (2)	0·7	2·0	Nil	97·3
		=N—	(5·5)					
29	Oxine-5-carboxylic acid	—COOH	(4·8)	(1), (2)	98·4 (—COO⁻) / 1·0 (—O⁻ + —COO⁻)	<0·1	Nil	0·6
		—OH	9·32	(1)				
		=N—	(4·0)	(1), (2)				
31	Oxine-5-sulphonic acid	—SO$_3$H	2·04	(1)	94·1 (—SO$_3^-$) / 5·9 (—O⁻ + —SO$_3^-$)	<0·1	Nil	Nil
		—OH	8·48					
		=N—	4·19					
32	7-Iodooxine-5-sulphonic acid	—SO$_3$H	<2	Feldman & Powell (1940)	44·3 (—SO$_3^-$) / 55·7 (—O⁻ + —SO$_3^-$)	<0·1	Nil	Nil
		—OH	7·15					
		=N—	2·45					
33	1-Hydroxyacridine	—OH	(9·0)	Albert & Goldacre (1943)	1·8	0·3	Nil	97·9
		=N—	(4·7)					
34	1-Methoxyacridine	=N—	(4·7)	Albert & Goldacre (1946)	Nil	0·3	Nil	99·7
36	1-Hydroxyphenazine	—OH	8·5	Michaelis, Hill & Schubert (1932)	5·9	<0·01	Nil	94·1
		=N—	1·44					
40	6-Hydroxy-*m*-phenanthroline	—OH	(8·5)	(1), (2)	5·9	<0·1	Nil	94·1
		=N—	(3·9)					
42	8-Aminoquinoline	=N –	3·93	Albert & Goldacre (1944)	Nil	<0·1	Nil	*c.* 100

(1) Specially determined for the present studies by Mr R. Goldacre and Mr J. Phillips.

(2) The pK values in brackets had to be calculated approximately from values obtained in 50% ethanol, because of the sparing solubility of the substances in water. The corrections of −1·7 and +0·5 were made to the —OH and =N – values respectively as was suggested by the pK values of oxine (10·92 and 4·57 respectively), and the acid pK of 7-chlorooxine (9·61), when determined in 50% ethanol.

(3) Determined in 50, 40, 30, 20 and 10% ethanol and extrapolated to pure water.

of cation varies from $<0{\cdot}1\,\%$ (several examples) to 29 % (no. 22) or even, if the ionization of a group other than the ring nitrogen is considered, 98 % (no. 26). Only two examples of substances which yield a large proportion of zwitterion are found in the series (nos. 22 and 25). Among the various members, the percentage of neutral molecule varies from nil to practically 100 %.

DISCUSSION

Interest in the biological properties of oxine goes back to about 1895 in which year the firm of Fritzsche and Co., Hamburg, brought out a product, Chinosol, for use as an antiseptic and disinfectant. The composition of this product was found by Brahm (1899) to be a mixture of 8-hydroxyquinoline and potassium pyrosulphate, the acidity of the latter helping to dissolve the former. The great dilution at which oxine acts against bacteria and fungi (often M/80,000, i.e. 1 part in 500,000) would suggest that its mode of action depends on some specific interference with a vitally important metabolic reaction. Until recently there has been no attempt to investigate the mode of action of oxine which has usually been classed with the phenolic disinfectants (e.g. Hata, 1932). However, it was later suggested that oxine exerts its antibacterial action by withdrawing essential trace metals from bacteria (Albert, 1943). This hypothesis has been largely substantiated not only for oxine but for all derivatives shown in the present work to have the property of chelating hydrophobically with metals (Albert *et al.* 1947). Zentmyer (1944) similarly explained the fungistatic power of oxine.

Little was known about the nature of the metallic complexes of oxine until Berg (1927) introduced it and some of its simpler derivatives into analytical practice for the determination of various metals. The reaction is very delicate (e.g. 1 μg. of zinc can be detected in 1 ml. of water, i.e. at a dilution of 1 p.p.m.), and is much used when traces only of metals are present. The ease with which small amounts of metal-oxine complexes can be shaken out of aqueous solutions by chloroform has increased the general utility of this reaction. Berg found that oxine precipitated different metals at different hydrogen ion concentrations and this differentiation was put on a scientific basis by Fleck & Ward (1933; also Fleck, 1937) who determined the pH limits between which metals would precipitate from M/300 solution, the majority of heavy metals being completely precipitable only between pH 4·5 and 14·5. However, magnesium was not precipitated at all below pH 7, to the extent of 3 % only at pH 7·7 and to the extent of 75 % at pH 8·5. The molybdyl, uranyl and tungstyl cations were precipitated completely only between narrow pH limits (e.g. pH 5·0–5·7 for tungstyl).

Mechanism of chelation by oxines

Theoretical considerations suggest that no isomeride of 8-hydroxyquinoline should be capable of chelation because the formation of a 5- or 6-membered ring, coplanar with the other rings, becomes impossible when the hydroxyl group is transferred to other positions in the quinoline nucleus. This postulate received some confirmation when it was found that iron, nickel, copper, palladium and platinum chelated with no hydroxyquinoline other than the 8-isomeride (Bargellini & Bellucci, 1923). That this is equally true of the metals investigated in the present work follows from the fact (Table 1) that oxine alone of its isomerides gave precipitates. Independent testing for hydrophilic chelation, as described in the foregoing text and exemplified by Fig. 4, substantiated the conclusion that oxine alone of the seven isomeric hydroxyquinolines is capable of chelation.

To provide a basis for the control of chelating power among derivatives of oxine, it becomes necessary to look more deeply into the mechanism of chelation. What is already known on this topic is summarized by Fig. 1, viz. that oxine complexes are formed by the metal (i) becoming linked to the oxygen atom through a primary valence and (ii) completing the ring by forming a co-ordinate bond with the nitrogen atom, utilizing the latter's lone pair of electrons for this purpose. Further, it is known that, in at least one example, the bond between the oxygen and the metal is more polar than covalent (Mellor & Craig, 1941). Concordant with these views are our findings that oxine will no longer chelate with metals when either the O or N is firmly blocked, as by a methyl group in nos. 9, 10 and 12 (see Table 2). By far the most likely first step in the formation of oxine-metal complexes is that the ion of the metal combines with oxine anions and not that the metallic ion breaks the O—H bond in the course of random collisions with the unionized hydroxyl group. Accordingly, factors which modify the ionization of the hydroxyl group in oxine could conceivably interfere with chelation. For example, a graded repression of ionization ought to be procurable by the introduction of electron-repelling groups (such as $—CH_3$, $—NH_2$ and —OH), particularly if these were to be placed in the ring containing the —OH group, and it was thought of interest to note whether these occasioned a proportionate drop in ability to chelate.

As the literature offered little information on the ionization of oxine derivatives, the examination of a number of our compounds from this point of view was arranged (Table 6). It was found that the electron-repelling effect of an alkyl group (as exemplified by 5-propyloxine, no. 14, Table 6), affected ionization in the expected direction to too slight an

extent to interfere with chelation (nos. 11, 13 and 14, Table 2). When more strongly electron-repelling groups (e.g. —OH and —NH_2; nos. 23 and 27) were inserted in the oxine molecule, the connexion between ionization and chelation was somewhat obscured by the susceptibility of the new compounds to aerial oxidation in the presence of traces of metals (e.g. zinc ions). For example, 5-aminooxine (no. 23) shows the expected marked decrease in ionization of the hydroxyl group and it appears not to chelate with metals in the manner of oxine, but it gives solutions of various colours in proportion to the amount of air admitted and the nature of the metal. Fortunately some other oxines with a low percentage of anion (i.e. $<0{\cdot}1\%$) at pH 7·3 are not so prone to metal-catalyzed oxidation (nos. 22 and 25; Tables 2 and 6). Here too, there is a virtual disappearance of chelating properties, e.g. no. 22 gives a slight precipitate with copper but does not precipitate or give the hydrophilic chelation test with zinc.*

On the other hand, no outstanding improvement in chelating properties seems to have been effected by increasing the percentage of anion present in oxines. Attempts to favour chelation by increasing the electron flow towards a participating nitrogen, as recommended by Calvin & Bailes (1946), can give equivocal results when, as here, the nitrogen can ionize at the pH of the experiment. In fact, until it is possible to determine stability constants for metal-oxine complexes (as discussed in Part I; Albert & Gledhill, 1947), treatment of all such questions can be no more than semiquantitative.

Some other factors involved when comparing the effects of substituents on the chelation of oxines will now be briefly referred to. Precipitation phenomena are affected, favourably or otherwise, by substituents which render the metal-oxine complexes less, or more, soluble. The alkyl and halogen derivatives of oxine in Table 2 provide several examples where precipitation can be recognized at a tenfold higher dilution than is the case with oxine. On the other hand, the insertion of a sulphonic acid group does not prevent chelation from taking place although the metal complexes become water soluble, an effect which has been described at length above. It should be noted that compounds of this type (nos. 26, 29, 31 and 32, Tables 2 and 6) have a peculiarity in that the chelating ion carries two charges. Perhaps the principal value of Table 6 lies in its demonstration that a highly complex ionic situation can arise when a few common substituents are introduced into the molecule of a potential ampholyte such as oxine.

The remaining compounds of Table 2 have some points of interest; 5:7-dinitrooxine (no. 20) has the hydrogen-bonded *o*-nitrophenol structure; 5-nitrosooxine (no. 21) is tautomeric with the monoxime of 5:8-quinolinequinone; tetrahydrooxine (no. 28) is catalytically oxidized in the presence of metals; such features may explain the lowered chelating powers of these compounds.

All the structural features responsible for the chelation of oxine can be reproduced in heterocyclic ring systems other than quinoline (e.g. nos. 33, 36, 39 and 40, Table 3). The chelating powers of 1-hydroxyacridine have already been recorded (Freeman & Lions, 1940); the other examples are new. As with oxine, ability to chelate is lost by isomerization (nos. 35 and 38) or by blocking the hydroxyl group (nos. 34 and 37). It may be added that pyocyanin, an *N*-methylated derivative of 1-hydroxyphenazine, does not chelate. The remaining compounds in Table 3 show that systematic departure from the arrangement of atoms responsible for chelation in oxine leads to diminishing degrees of ability to chelate.

One of the most interesting findings of this survey is that with very few exceptions oxine, and substances similarly constituted, chelate with the same selection of metals. Disregarding those substances which are destroyed by air in the presence of metals (nos. 22–28 inclusive, 46 and perhaps 21), one sees a tendency to chelate with a limited range of metals only in 5-nitrooxine (no. 19, Tables 2 and 4) and 1-hydroxyphenazine (no. 36, Table 3). Even here there is not the high specificity that often appears in other classes of chelating compounds (e.g. nos. 41 and 45). Throughout the entire series, no case occurred where calcium or magnesium was precipitated at pH 7·3. The colours of the complexes formed by iron and various oxines were always blue or green in contrast to those of other metals which partook of the colour of the chelating agent (usually pale yellow).

Biological and biochemical implications

The data presented above have a bearing on the use of oxines as drugs in chemotherapy and as reagents in biological research. Although the antibacterial action of oxine has been known so long and so many modifications of the molecule have been made (Altpeter, 1929), it had been difficult to

* In nos. 22 and 25, the phenomenon of extra ionic resonance, associated with the presence of amino groups in certain positions (Albert & Goldacre, 1944) has caused the basic centres to become ionized at the pH studied. In these compounds the positive charge, by attracting electrons, increases the ionization of the hydroxyl groups, but the net effect has been to decrease the amount of available anion owing to its inevitable participation in zwitterion formation through internal neutralization. In no. 26, which chelates hydrophilically owing to the diguanide cation being a powerful water-attracting group, the cationic species (present to the extent of 98%) is free to chelate as the ring nitrogen does not carry a charge.

visualize the possibilities and limitations of this series until the mode of action was discovered. In a collateral publication (Albert *et al.* 1947) it is shown that the antibacterial action of oxine derivatives runs remarkably parallel with their ability to chelate and hence bacteriostasis probably depends on the removal of trace metals which are essential to bacteria. Certain cases, where inert groups in particular positions interfere with antibacterial action without interfering with chelation *in vitro*, are discussed from the viewpoint of steric hindrance and it is concluded that the antibacterial action occurs, not in solution, but at a bacterial surface.

For use as a biological tool, to remove a catalytic or a protective metal, the value of any oxine can be roughly gauged from the present work. The comparative lack of metal specificity is unfortunate but it may be permissible in some cases to remove the oxine-metal complexes by shaking out with chloroform and when this can be done at various pH values, a degree of metal specificity is attainable (Moeller, 1943). We have observed that chloroform does not remove the metal complexes of amino-acids (e.g. glycine, arginine, tryptophane). As it is known that some metals which catalyze the oxidation of thiol groups can do so even when bound to oxine (Bernheim & Bernheim, 1939), one must not regard metal complexes as being necessarily inert and their removal (e.g. by chloroform) may be imperative in some types of work.

SUMMARY

1. Forty five analogues and derivatives of oxine (8-hydroxyquinoline) have been investigated with reference to their ability to combine, by chelation, with trace metals in neutral solution.

2. It was found that oxine alone of the seven isomeric hydroxyquinolines had chelating ability and that this property persisted throughout all derivatives having the essential chelating mechanism of oxine, viz. a phenolic group *peri* to a heterocyclic tertiary nitrogen atom. Chelate activity was found to be partly or totally suppressed in proportion as this arrangement of atoms was modified.

3. Several derivatives of oxine were found to give precipitates with metals at a greater dilution than did oxine. No outstanding instances of specificity for any metal were encountered.

4. The somewhat complex influence of electronic distribution, and hence ionization, on chelating in this series is briefly discussed.

5. Some applications of the experimental results to problems in biology and biochemistry are indicated.

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The Potentiation of the Toxicity of some Vesicants to the Pyruvate Oxidase System *in vitro* by certain Dithio-compounds

By R. A. PETERS and R. W. WAKELIN, *Department of Biochemistry, Oxford*

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One of the more puzzling features of the *in vivo* action of mustard gas has always been the small amount which can prove toxic to animals. In this laboratory, for instance, it was reported (Holiday, Ogston, Peters, Philpot & Stocken, 1940) that 2 mg. of mustard gas placed upon the skin of most rats weighing 200 g. would kill. Allowing for loss by evaporation, hydrolysis and fixation in the skin, this means that the toxicity is < 10 μg./g. of rat, and is of a different order from that associated with the *in vitro* action of mustard gas upon certain enzymes and enzyme systems which is *c.* 300 μg./ml. (Peters & Wakelin, 1940; Dixon & Needham, 1941*a*; van Heyningen, 1941); it is also quite different from the toxicity of arsenicals and of divinyl sulphone and 2:2′-dichlorodiethyl sulphone, which approaches *in vitro* more closely the concentration of *in vivo* toxicity. There is much evidence now of causal connexion between lewisite action and enzyme poisoning (see review by Peters, Stocken & Thompson, 1945). It would be satisfactory also to interpret the activity of mustard gas in terms of an enzyme poisoning; Dixon, van Heyningen & Needham (1942) have indeed developed the theory that mustard gas and other vesicants owe their action to toxic effects upon hexokinase. In the course of this work, in which the pyruvate oxidase system was used as a test for possible thiol-containing therapeutic agents (Holiday *et al.* 1940), it was found that *N*:*N*-diethyldithiocarbamate or *N*:*N*-di-(2-hydroxyethyl)-dithiocarbamate had a special action. Instead of protecting the pyruvate oxidase system against mustard gas it much increased its toxicity; this effect was called 'potentiation'. The important point was made that in the presence of some dithio compounds, mustard gas becomes almost 20 times more toxic, and some evidence was produced that the action was due to a compound of the dithio substance and mustard gas. While there is no reason to think that the potentiating action of these dithio compounds occurs *in vivo*, it was felt that a closer study was advisable. We were reinforced in our suspicion that there might be unsuspected ways in which the action of mustard gas was altered *in vivo* by our finding that mustard gas was often toxic to the succinic dehydrogenase enzyme in a brain *brei*, whereas it was not so in the same enzyme in ground-muscle preparations (Peters & Wakelin, 1941). The opposite is true of phosphocreatine phosphokinase, which is sensitive to mustard gas in the isolated state (Cori, Colowick, Berger & Slein, 1945), whereas it has not been found to be poisoned by treating skin with mustard gas *in vivo* (Needham, quoted by Dixon, 1943).

Following our initial observations, Dixon & Needham (1941*b*), using the pyruvate oxidase of *E. coli*, confirmed the effect of diethyldithiocarbamate, and the toxicity of the incubated product; they added the further observation that addition of diethyldithiocarbamate (not itself toxic), after hydrolysis of mustard gas was complete, could still cause an inhibition. Hence they thought that the potentiations could not be explained simply by the formation of a more toxic addition compound.

In this report, modified from the original (Peters & Wakelin, 1943*b*), we present a closer study of the potentiating effect upon the pyruvate oxidase system of diethyldithiocarbamate and diethanoldithiocarbamate; no essential difference in the action of the two compounds has been found. The toxic effects are due to the formation of a compound of mustard gas with the diethyldithiocarbamate ion, which is more toxic than the sum of the toxicities of the two compounds.

EXPERIMENTAL

Brei from pigeon brain has been used as described previously (Peters & Wakelin, 1946). We have determined the amount of the compound giving a 50% reduction in O_2 uptake in presence of pyruvate over the respiration period 30–120 min. from the start of the readings. When diethyldithiocarbamate (*D*) or diethanoldithiocarbamate (*Dol*) was used, this was put into the respiration bottles before the poison. Both *D* and *Dol* have some toxicity; with *brei*, we used 1 mg./ml. and with dispersions, 0·5 mg./ml. Where dialyzed dispersions were employed, the dialysis was carried out for 4–5 hr. at 2° against 0·4% KCl. Q_{O_2} values have been calculated assuming that pigeon brain contains 80% water. In the case of *brei*, during the respiration period of 30–120 min., the residual respiration becomes much reduced and is therefore relatively less important as the experiment proceeds; further, since the presence of pyruvate inhibits lactate oxidation (Green & Brosteaux, 1936), there will be less residual respiration in the bottles containing pyruvate; any traces of pyruvate formed from residual lactate will not increase the O_2 uptake as the system is already saturated with pyruvate. In our earlier experiments we always set up controls for the residual respiration; but based upon the considerations above and upon experimental observations, we dispensed with these in this series and changes from the results with pyruvate alone were calculated. Much gain in time and saving of animals result when pyruvate is added to all twelve of the bottles, as one control and five duplicate observations are possible on one brain. Any toxicity of the dithiocarbamate has been allowed for by subtracting this from the total toxicity figures.

The procedure originally reported (Holiday *et al.* 1940) for obtaining maximal increases in toxicity has been used. Diethyl- (or diethanol-) dithiocarbamate (1 mg./3 ml. of Ringer-phosphate) was added after division of the *brei* or after adding the dispersion to the bottles; after shaking for 1–2 min., the mustard gas emulsion in lecithin was then added. Under these conditions the extra toxicity will be observed within 10–15 min. and will be at a maximum after 30 min. at 38°. The reasons for using lecithin and the arguments indicating that it has no adverse influence on the results are given fully elsewhere (Peters & Wakelin, 1941).

Mono- and bis-compounds of mustard gas and diethyldithiocarbamate were tested. The preparation of the mono-compound (I) 2-chloroethyl 2-(*N*-diethyldithiocarbamato)-ethyl sulphide and bis-compound (II) bis-[2-(*N*-diethyldithiocarbamato)-ethyl] sulphide is described elsewhere (Peters & Wakelin, 1947).

Compound (I) $(C_2H_5)_2N.\overset{\overset{S}{\|}}{C}.S(CH_2)_2S(CH_2)_2Cl$

Compound (II) $(C_2H_5)_2N.\overset{\overset{S}{\|}}{C}.S(CH_2)_2S(CH_2)_2S.\overset{\overset{S}{\|}}{C}.N(C_2H_5)_2$

RESULTS

Potentiation

Table 1 shows the potentiating effect of *Dol* upon the toxicity brought about by mustard gas and some other compounds. It will be seen that there is approximately a twenty-fold increase in the toxicity of mustard gas, and smaller increases also occur with other compounds. In Table 2, the result of addition of *Dol* upon several compounds having reactive Cl atoms as in mustard gas itself are compared at a concentration of 200 μg./3 ml. At this concentration the effect of mustard gas added alone is very slight. It will be noticed that the effect is larger with the compounds containing the two reactive Cl atoms.

Table 1. *The effect of diethanoldithiocarbamate* (Dol) *upon the concentrations of poisons required to produce* 50% *inhibition of* O_2 *uptake in the pyruvate oxidase system*

(Pigeon brain *brei*; substrate, Na pyruvate, 0·018 M; results the average for respiration period 30–120 min., 38°.)

		With *Dol* (μM)	No *Dol* (μM)
Arsenical	Lewisite	16	16
Non-arsenical	Iodoacetamide	(60)*	60
	Divinyl sulphone	(67)†	67
	Mustard gas	(63)‡	>1000
	2:2′-Dichlorodiethyl sulphone	110§	110
	2:2′:2″-Trichlorotriethylamine	130‖	750
	Allyl *iso*thiocyanate	195¶	242¶

* One determination gave for 18 μM, −24% change in respiration.

† One determination gave for 33·5 μM, −28% change in respiration.

‡ Based upon 14 determinations (10 different brains) at different concentrations of mustard gas.

§ One determination.

‖ Three determinations.

¶ Three determinations; these values are approximate but they indicate the order of toxicity. The difference of results with and without *Dol* is not significant.

(Results were calculated on total oxidation with pyruvate as substrate, without subtraction of the residual respiration.)

Conditions for potentiation. Table 3 gives a list of all substances tested and shows that only substances containing the dithio grouping have this action; it was not found with all of these. The list in Table 3 shows that potentiation was confined to substances which were capable of alkylation reactions similar to mustard gas. The absence of potentiation with the arsenical tested, or with the sulphones, is interesting, and also the fact that, in the presence of *Dol*, the toxicity of mustard gas to the pyruvate oxidase system approaches that of divinyl sulphone.

Nature of the potentiation. This has been investigated in the case of mustard gas only and diethyldithiocarbamate (*D*). The toxicity is due to some compound other than mustard gas, for the following reason. If an emulsion of mustard gas (*c.* 1 mM) be shaken in Ringer-phosphate for approx. 45 min. at 38°, it is then practically non-toxic, owing to hydrolysis to thiodiglycol and HCl; but an emulsion of

Table 2. *The effect of diethyldithiocarbamate* (D) *upon the toxicity of sulphides containing* Cl *as in mustard gas compared at concentrations of* 200 μg./3 *ml.*

(Substrate Na pyruvate.)

Compound	Remarks	Change in respiration (%)		Change due to *D* (%)
		No *D*	With *D*	
2-Chloroethyl ethyl sulphide	One Cl	−28	−38	−10*
2-Chloroethyl propyl sulphide	One Cl	− 5	−42	−37†
2-Chloroethyl phenyl sulphide	One Cl	−10·5	−40·5	−30‡
2:2′-Dichlorodiethyl sulphide	Two Cl	−13	−80	−67
Di-(2-chloroethylthioethyl) ether	Two Cl	−(0–7)	−(52–56)	−(45–56)§
1:1′-Dichlorodiethyl sulphide	Two Cl	−17	− 7	+10
3:3′-Dichlorodipropyl sulphide	Two Cl	−23	−38·4	−15

* 78 μg. alone gave −2% change in respiration.
† 86 μg. alone gave −16%.
‡ 108 μg. gave nil to 11·5%; +*D* −22%.
§ 50 μg. (63 μM) alone gave −23% and −36·5%; 37 μg. gave −35%.

Table 3. *Substances which potentiate or do not change the effect of mustard gas, when added together with this to a* brei *or dispersions of pigeon brain*

Potentiating	No change
Diethyldithiocarbamate	2:3-Dimercaptopropanol (BAL)
Diethanoldithiocarbamate	Thiosalicylate } Both improve respiration
Diethanedithiophosphonate	*p*-Thiol benzoate }
Butanedithiophosphonate	Monothiophosphonates (Na salts) } Methane, ethane, *n*-butane, toluene, diethane, *n*-hexane
	Dithiophosphonates (Na salts) } Methane, ethane
	Na ethyl monothiocarbamate
	Thiomalic ester
	Allyl *iso*thiocyanate
	Na thiosulphate

Table 4. *Substances potentiated by diethyl- (or diethanol-) dithiocarbamate*

Effect	Potentiated
Large	Mustard gas
	Di-(2-chloroethylthioethyl) ether
Intermediate	2:2′:2″-Trichlorotriethylamine
Small	2-Chloroethyl ethyl sulphide
	2-Chloroethyl propyl sulphide
	2-Chloroethyl phenyl sulphide
	2-Chloro-2′-hydroxydiethyl sulphide
Slight	3:3′Dichlorodipropyl sulphide

mustard gas added to *D* in Ringer-phosphate is still toxic after 2 hr. and will retain this toxicity after storage in the cold. Exp. 2373 (Table 5) shows this; exp. 2374 shows that the compound is much more toxic than mustard gas itself, and that emulsification is necessary for full development of the toxicity. The toxic action develops quickly, and the greatest toxicity is obtained by warming the lecithin mixture of the compound and centrifuging in Ringer-phosphate at 38°.

By shaking mustard gas in an aqueous solution of *D* under conditions simulating those of the potentiation experiment, a mono-substitution product (I) is formed, which has been isolated as crystalline needles m.p. 40·5° (Peters & Wakelin, 1943*a*, 1947). The compound is soluble in c. 60% ethanol, and by kinetic methods (Peters & Walker, 1923) the rate of acid liberation is the same as that of mustard gas in the same solvent.

Table 6 shows the action of the highly purified compound (I) when compared with an amount of *D* equivalent to that contained in the concentration of (I) tested.

Table 7 shows that compound (I) does not further potentiate mustard gas and in its presence the latter shows no added activity; also that the bis-compound (II) of mustard gas and 2 molecules of diethyldithiocarbamate is not toxic under these conditions with a *brei*, and that mustard gas exerts its usual toxic effect in its presence.

Causes of potentiation

Since the mono-compound (I) is toxic, and the bis-compound not so, and since (I) still contains a Cl capable of substitution, it was felt first that potentiation must be due to the formation of the tissue compound (III) from (I).

This view was not entirely tenable because (*a*) the crude but Cl-free OH compound (IV) found by hydrolysis of (I) was still toxic and (*b*) the mono-Cl analogues of mustard gas showed potentiation,

Table 5. *Toxicity of compounds (not isolated) formed by shaking mustard gas with diethyldithiocarbamate* (D), *and effects of emulsifying with lecithin*

(Pigeon *brei*; substrate, Na pyruvate.)

Exp. 2373. Toxicity of mustard gas (lecithin emulsion) after shaking with diethyldithiocarbamate for 70 min. at 38° in respirometer bottles.

Addition	Q_{O_2} 30–120 min.	Decrease (%)
Equivalent of *D* alone (150 μg.)	10·72	—
Product of *D* + 200 μg. mustard gas	6·41	40

Exp. 2374. Toxicity of mixture of *D* + mustard gas (*c.* equimolar) when presented to the brain tissue as a suspension or as an emulsion in lecithin. (Lec. = lecithin. Na pyruvate in all bottles and diethyldithiocarbamate added in equivalent amounts as control where necessary. The mixture of Lec. + *D*, Lec. + *D* + mustard gas, *D* + mustard gas, and *D* alone were shaken respectively for 1 hr. at 38° in Ringer-phosphate solution. Samples (0·4 ml. ≡ 200 μg. mustard gas) were then added to the experimental bottles either suspended or re-emulsified with lecithin.)

	Addition	Q_{O_2} (0–30 min.)	Q_{O_2} (30–120 min.)	Change (%) (30–120 min.)
Suspended	*D* alone	14·55	10·57	—
	Product (*D* + 200 μg. mustard gas)	12·70	9·90	− 6
	Product (*D* + 800 μg. mustard gas)	12·50	9·13	−14
Emulsified	Lec. + *D*	13·42	9·67	—
	Lec. + product (*D* + 200 μg. mustard gas)	8·24	5·94	−38·5

Table 6. *Comparison of toxicity of crystalline compound* (I) *of mustard gas and diethyldithiocarbamate with an amount of the latter equivalent to that combined in the amount of* (I) *used for test*

(Brain *brei*. Na pyruvate, 0·018 M.)

Addition	Q_{O_2} (30–120 min.)
Lecithin only	10·22
Lecithin + *D* (191 μg.)	9·37
Lecithin + (I)* (300 μg.)	5·58

* (I) emulsified with lecithin at 38° by mixing and stirring into warmed Ringer-phosphate solution.

though to a much less extent than the bis-Cl compound.

Compound (III) $(C_2H_5)_2N.C(=S).S(CH_2)_2S(CH_2)_2$-tissue enzyme

Compound (IV) $(C_2H_5)_2N.C(=S).S(CH_2)_2S(CH_2)_2OH$

We think that the non-toxicity of compound (IV) is largely due to its failure to reach the active centre; even when emulsified with lecithin, it is largely present as crystals at 38° and remains suspended. This view is supported by other work, because very toxic effects have been obtained (Table 8) with a non-crystalline compound of diethanoldithiocar-

Table 7. *Comparison of toxicity of mono- and bis-compounds* (I) *and* (II) *in presence and absence of added mustard gas* (MG)

(Brain *brei*. Na pyruvate, 0·018 M.)

	Addition	Q_{O_2} (0–30 min.)	Q_{O_2} (30–120 min.)	Change (%) (30–120 min.)
Exp. 2382.	Lec. only	14·0	10·03	—
	Lec. + 0·6 mg. (I)	9·01	3·94	− 61
	Lec. + 0·6 mg. (I) + 0·33 mg. MG	9·06	3·72	− 63
	Lec. + 2 mg. (II)	14·13	10·51	+ 5
	Lec. + 2 mg. (II) + 0·33 mg. MG	12·22	7·59	− 27
Exp. 2383.	Lec. only	13·02	10·93	—
	Lec. + 0·6 mg. (I)	7·85	3·55	− 67
	Lec. + 0·6 mg. (I) + MG	8·39	3·19	− 71
	Lec. + 1 mg. (II)	13·02	10·38	− 5
	Lec. + 1 mg. (II) + MG	12·71	7·89	− 24

Lec. = lecithin.

bamate and mustard gas free from Cl, which, though impure appeared to be mainly the bis-*Dol* compound, and which was much more soluble than the diethyl compound.

Table 8. *Toxicity of bis-product of* Dol + *mustard gas*

(Brain *brei*. Na pyruvate, 0·018 M.)

Addition	Q_{O_2} (30–120 min.)	Change (%)
Lecithin only	9·80	—
Lecithin + 300 μg. (*Dol* only)	8·85	−10
Lecithin + 300 μg. (*Dol* + MG)	2·61	−73

Hence the conclusion is reached that it is the $=N.C\begin{matrix}S\\ S-\end{matrix}$ grouping in compound (I) which is inherently toxic; this would be in agreement with the lesser toxicity of dithiocarbamates themselves and of allyl *iso*thiocyanate. Substitution in the SH thus increases the reactivity of the thio (=S) group.

In the potentiation experiment, the effect will be due to a combination of (*a*) the toxicity of compound (I) or its OH derivative and (*b*) formation of tissue compounds as (III) by substitution of Cl either directly or through compound (I) as intermediary. The latter provides a possible explanation of Dixon & Needham's (1941*b*) experiments; in their case, the initial treatment with mustard gas may have left unhydrolyzed Cl half-products such as (V) available for combination with added *D*. We have shown already that such products are present in mustard gas-kerateine (Peters & Wakelin, 1941).

Compound (V):

Tissue enzyme-$(CH_2)_2S(CH_2)_2Cl$ + Diethyldithiocarbamate

$$\longrightarrow \text{Tissue enzyme-}(CH_2)_2S(CH_2)_2S.\overset{\overset{S}{\|}}{C}.N(C_2H_5)_2$$

In reaching the above conclusions, we have discarded some other possibilities, which may be mentioned briefly.

Discarded possibilities. We have produced evidence for the presence of SH groups in brain pyruvate dehydrogenase (Peters & Wakelin, 1946; circulated privately in 1941) from experiments with S—S compounds, and by showing that sodium maleate is inhibitory in very low concentration. It might be thought that potentiation was produced by blocking some of the SH groups necessary for pyruvate dehydrogenase action, and that this might render the whole system more sensitive. In the experiments of which one is quoted (Table 9) with various concentrations of maleate no potentiation was observed. This does not suggest that potentiation is due to partial blocking of an SH group.

Again, it was thought that the SH groups might be partly oxidized to S—S by oxidized diethyldithiocarbamate, as Keilin & Hartree (1940) have demonstrated an inhibition of this nature with succinic dehydrogenase. In a test, however, we could obtain no inhibition in this way. Again, potentiation cannot be reversed either with glutathione or with kerateine (the latter is known to stabilize respiration, see Peters & Wakelin (1941)).

Table 9. *Effects of* Na *maleate and mustard gas upon* Q_{O_2} *of brain* brei *with pyruvate*

Addition/3 ml.	Decrease in Q_{O_2} (%)	Total decrease (%)
200 μg. mustard gas	− 8	−46
400 μg. Na maleate	−38	
200 μg. mustard gas + 400 μg. Na maleate	−46·5	−46·5

Toxicity to animals

When impure, compound (I) is toxic to the rat, both as applied on the skin and by injection, and kills with the signs of mustard gas poisoning, except that there is a tendency for ulceration of the skin, which is unusual for mustard gas. Dr Holiday tells us that he once observed this also with a crude mixture. It is undoubtedly due to residual traces of mustard gas or its half-hydrolysis product dissolved in the oil. We have found these crude oils very irritant to mustard gas-sensitive men. When pure we have injected comparatively large doses, emulsified in lecithin, intraperitoneally into several rats and one guinea-pig. In two cases, the rats died after 1 and 2 months respectively, but we hesitate to regard this as significant. If placed in the eye of a rabbit, no ill effects have followed (Mann & Pirie, 1943).

Since the compound is toxic to the pyruvate system *in vitro*, it is evident that its physical properties prevent penetration to the active centres *in vivo*, probably owing to predominant lipid solubility. A similar conclusion was reached for the difference in toxicity between some acriflavines (Manifold, 1941).

DISCUSSION

The much greater potentiation shown with the di-Cl compounds is the first distinction between mono-Cl and di-Cl consistent with competition reactions (Holiday, Ogston, Philpot & Stocken, 1940), for which there is the explanation that the greater activity may be due to the possibility that more than one toxic compound can be formed with the di-Cl compound. In regard to the action of the substituted dithiocarbamate compound, one possible view is that it diverts an attack upon some other grouping into one upon SH groups in the pyruvate oxidase system.

So far there has been little exploration of the enzyme field. We have found that inhibition of the succinoxidase system by mustard gas, when it occurs, is not potentiated by *D*. As the cytochrome system is common to both this and the pyruvate system, this means that it is not the cytochrome component of the pyruvate system which is attacked. The potentiation can be observed also with brain dispersions.

SUMMARY

1. Some dithiocarbamates and dithiophosphonates potentiate the toxic action of mustard gas upon the pyruvate oxidase system; the effect of diethyl- (or diethanol-) dithiocarbamate has been closely examined.

2. Only compounds having a Cl atom which behaves kinetically as in mustard gas are potentiated; the effect is quickly established.

3. Potentiation is due to the formation of the half product of mustard gas with diethyldithiocarbamate (compound (I), 2-chloroethyl-2-(*N*-diethyldithiocarbamato)-ethyl sulphide), prepared crystalline under the conditions of the experiment. This is much more toxic than either of its components.

4. The toxicity of (I) is not due to the Cl group; the hypothesis is advanced that the $=N.C\begin{matrix}S\\S-\end{matrix}$ group is inherently toxic.

5. The bis-substitution product was only slightly toxic, which is explained by its inability to reach the active enzyme centre in the brain *brei*.

6. (I) is not toxic when injected into rats. This is interpreted as due to failure to reach the pyruvate system *in vivo*.

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Observations upon a Compound of Mustard Gas and Kerateine

By R. A. PETERS and R. W. WAKELIN, *Department of Biochemistry, Oxford*

(*Received* 20 *December* 1946)

The fact that mustard gas will combine with some proteins is known from the work of Berenblum & Wormall (1939) on serum proteins, Berenblum (1940) on nucleoproteins, Pirie (1941) on collagen, and may be inferred also from the work of Fell & Allsopp (1940–1). Since enzymes are proteins, such combination is indicated from enzyme studies in Oxford (Peters & Wakelin, 1940) and Cambridge (see summary by Dixon & Needham, 1946), as also from kinetic considerations of the reactions of mustard gas (Holiday, Ogston, Philpot & Stocken, 1940; Ogston, 1941).

Earlier work in this laboratory (Peters & Walker, 1924–5) had shown that mustard gas did not abolish the fixed SH reaction (Hopkins & Dixon, 1922) of extracted muscle tissue though dichlorodiethyl sulphone and phenyldichloroarsine readily did so. This is rather puzzling because mustard gas so readily combines with simple monothiols (Holiday *et al.* 1940; van Heyningen, 1941). The original object of

this work (Peters & Wakelin, 1941) was partly to determine whether mustard gas combines with the SH groups in protein products containing these and partly to provide material for experiment upon attempts to reverse any such combination.

EXPERIMENTAL

Kerateine, the soluble SH protein formed from keratin (Goddard & Michaelis, 1934) by reduction with alkaline cyanide, was used, owing to its relatively high content of SH and its successful use by Stocken & Thompson (1940, 1946). Upon the advice of Dr Thompson we used wool instead of human hair, which gave almost colourless products. The mixture containing the wool (100 g.) after digestion for 2–3 days in alkaline cyanide at 38° was acidified with acetic acid to pH 5·0±0·3. The ppt. was removed on a hardened filter paper, washed once with water and stirred into 1·7 M-phosphate buffer (pH 7·4), alkali being added to bring the solution to pH 7·4 (total vol. *c.* 400 ml.). Glass-distilled water was used throughout to exclude the oxidizing effect of traces of metals. To reduce risk of oxidation of SH groups, the precipitation and solution at pH 7·4 were left until the last; 100 ml. were removed before proceeding further to act as control. The remaining 300 ml. were stirred in a flask under N_2 and treated with mustard gas dissolved in absolute ethanol (14% v/v or *c.* 20% w/w). This solution (25–30 ml. containing 5–6 g. mustard gas) was gradually stirred in over 0·75 hr. at 25°; slow stirring was continued for *c.* 1 hr. At the beginning, small samples gave a strong immediate nitroprusside reaction; later this became rather slight, and not much increased by cyanide addition, indicating a genuine reduction in SH-yielding groups. The final pH was about 7·0. The preparation was cooled and brought to pH 4·8 with acetic acid; the ppt. was filtered off, washed with 0·01 N-acetate at pH 4·8 and then with successive portions of acetone; the first acetone washings usually contained detectable amounts of mustard gas. When there was no substance forming acid in presence of water in the acetone washings, the ppt. was transferred to a centrifuge tube, washed again with acetone and finally dried *in vacuo*. The final product was a fine, white, fluffy powder. In order to free it from the last traces of inorganic chloride under conditions least likely to affect the hydrolyzable organic chlorine, the product was purified by stirring up in minimal amounts of ice-cold water to which *c.* 2·5 ml. 20% NaOH were gradually added to bring to pH 7·4 (if moistened first with gradual addition of alkali, a better solution was obtained); the volume was finally adjusted with ice-cold water to 100–150 ml.; *c.* 1·8 ml. glacial acetic acid were then added to reprecipitate the 'mustard gas-kerateine' (pH 4·5–5·0). The ppt. was centrifuged in two 100 ml. tubes, washed first with dilute acetic acid (2 drops glacial acetic acid in 800 ml. water, once with acetone (100 ml.) and stirred with cool acetone for at least 2 hr., before drying after a further extraction with 100 ml. acetone.

In our experience, the final extraction by stirring in cold acetone is essential to free from the last traces of mustard gas, and Soxhlet extraction with ether is not so effective. Some preparations which had been well extracted with acetone, proved to be still irritant to the arm of a sensitive observer when 20 mg. powder was worn under cellophan for 2 hr. on the forearm. After thorough extraction by stirring, for a fresh extracted preparation, this irritancy disappeared.

Eight preparations were made upon the above lines, the last two by the exact technique described above; the yields of mustard gas-kerateine have varied from 15 to 20 g./100 g. wool. The control preparations were precipitated in a similar way from solution containing an amount of NaCl equivalent to the total amount of hydrolyzable Cl available from the mustard gas added in the 'mustard gas-kerateine' samples, and then worked up in an exactly similar manner.

RESULTS

S *and* Cl *content.* In all cases, the S content of the treated protein did not increase beyond twice that of the control. While the Cl content of the control has been practically negligible, it has been found as high as 0·36% in the 'mustard-treated' kerateine. Analytical values by Carius's method are given in Table 1 for the last two experiments; Exp. K must be considered the more accurate.

Table 1. *Sulphur, chlorine and nitrogen content of kerateine and mustard gas-kerateine*

(Results as % of protein wt., 0·5 g. samples used for analysis.)

Preparation		Mustard gas-kerateine	Control kerateine	Difference	Ratio (extra S/extra Cl)
G	Cl	0·355*	0·03	+0·325	—
	S	2·23	1·38	+0·85	2·9
K	Cl	0·349	0·022*	+0·329	—
	S	2·29	1·21*	+1·08	3·6
	N	14·3	14·9	−0·6	—

* Average of duplicate estimations.

The S content of the final product seems to approach 2·3% and the Cl content 0·35% rather constantly under these conditions; about one-third of the extra S must be present in the form of the 'half product' (kerateine-$CH_2CH_2SCH_2CH_2Cl$).

Porphyrindin titration of kerateine and mustard gas-kerateine. A more quantitative proof of the diminution of SH and S—S groups in mustard gas-kerateine is obtained by titrating with porphyrindin (1 mg./ml., standardized with cysteine ester) after conversion of S—S to SH in the protein by reduction with monothioethylene glycol at pH 7·3.

After solution at pH 7·3, the protein was treated *in vacuo* in 0·25 M-phosphate buffer for 2 hr. at 38° with monothioethylene glycol (20 mg./ml.); it was then rapidly reprecipitated at pH 4·5 by addition of N-HCl, and washed with dilute acetic acid until the centrifugate no longer gave the SH reaction (four washings necessary). NaCl (15%) at pH 4·5 (acetic acid) had to be used at this stage to keep the protein out of solution. When washing was complete, the protein was quickly redissolved in phosphate buffer (pH 7·2) and titrated with porphyrindin until the nitroprusside reaction disappeared. All the solutions were made with glass-distilled water, boiled and cooled before use to remove

O_2. The porphyrindin and cysteine ester were as used by Stocken & Thompson (1940) and kindly supplied by Dr Stocken.

Table 2 gives the last of three experiments, which consistently showed that the mustard gas-kerateine contains definitely less S—S+SH groups than the control.

Table 2. *Total* SH *groups present in kerateine and mustard gas-kerateine as determined by porphyrindin titration*

(Results before and after treatment with monothioethylene glycol.)

	Sulphydryl-S (%)	
	Before treatment	After treatment
Kerateine	0·12	0·34
Mustard gas-kerateine	Nil	0·11

The conditions of reduction were more severe than those of Mirsky & Anson (1934) for egg albumin; they used thioglycollic acid at 30° for 45 min. The value obtained by Stocken & Thompson (1940, 1946) for SH initially present was *c.* 0·39%, approaching our value of 0·34%. Nitroprusside reactions in presence of cyanide, which therefore include S—S groups, were in agreement with the titration values. We interpret this to mean that about one-quarter of the mustard gas has combined with available SH groups; the remainder must be in combination with other groupings. Some of the kerateine sulphur will be unavailable initially owing to conversion to —SCN by the cyanide used in preparation. (Since these experiments were completed, there is evidence in the work of Northrop (1943–4), Bailey, Banks, Boursnell, Francis, Webb, Wormall & Dixon (1945), and Banks, Boursnell, Francis, Hopwood & Wormall (1946) using radioactive sulphur, that mustard gas combines with carboxyl groups in proteins.)

The presence of thio-ether-linked mustard gas was confirmed by the use of the new silver reaction (Peters & Wakelin, 1942, 1947); mustard gas-kerateine gave an intensification of the cyanide nitroprusside reaction after silver treatment, which was not given by kerateine. Furthermore, from a fraction obtained after enzymic digestion (Exp. 1 below) the silver reaction indicated the presence of thio-ether-linked sulphur.

Experiment 1. Mustard gas-kerateine was thoroughly digested with pepsin followed by trypsin, giving a soluble preparation not entirely free from biuret-yielding substances. The digest was first treated with cuprous chloride in acid solution to precipitate S—S and SH compounds (following Hopkins's method for isolating glutathione as applied in quantitative work by Roussouw & Wilkin-Jorden (1935)); after removal of excess Cu with H_2S, the filtrate was treated with mercuric acetate at pH 4·5 according to the standard technique for precipitating methionine (Hill & Robson, 1934). After removal of Hg from the ppt. with H_2S, the substance present, which was not further isolated, resembled mustard gas-cysteine giving the faint cystine-like reaction with cyanide and a strong 'silver' reaction (type (*e*) reaction of Peters & Wakelin (1947)); the filtrate was inactive. No Sullivan reaction (for cysteine and cystine) was given by this fraction from the mercuric ppt. even after an acid hydrolysis, though glutathione when similarly hydrolyzed gave the reaction strongly. It was found that care had to be taken not to treat the thio-ether fractions with the metallic precipitant in neutral solution, otherwise it tended to give cystine-like reactions.

Reactive chlorine in mustard gas-kerateine. The presence of Cl in organic combination was shown by hydrolysis experiments.

Two 2·0 g. samples of mustard gas-kerateine were dissolved in 50 ml. ice-cold water at pH 7·4, and incubated at 38° for 1 and 3 hr. respectively. They were then cooled, reprecipitated and dried with acetone as before. Analytical values for Cl are shown in Fig. 1. These values confirmed and extended a previous experiment in which a similar treatment of 2 hr. had reduced the Cl content of the ppt. to 30% of the original. It seems clear that the Cl present is hydrolyzable, but much more slowly than the Cl of mustard gas itself (Peters & Walker, 1923); all would not be expected to have disappeared at 38° under 6 hr.

From the above we feel justified in concluding that in addition to any kerateine-$CH_2CH_2SCH_2CH_2OH$ there is an organic chloride present in mustard gas-treated kerateine, and that this is most likely to be a slowly hydrolyzing half product: kerateine-$CH_2CH_2SCH_2CH_2Cl$. From this it follows that fairly stable half products of mustard gas with proteins can be formed, and must be reckoned with in a final picture of the action of mustard gas on tissues, because they could link up over a period of hours with other protein tissue components.

Properties of mustard gas-kerateine. The powder almost completely dissolves at pH 7·4 to form an opalescent solution, provided that it is dissolved as recommended. The control kerateine is a similar white powder, more completely soluble at pH 7·4. Using as a measure the liberation of N not precipitated by trichloroacetic acid, both were digested by pepsin at equal rates, so that no linkage attacked by pepsin has been upset in the combination with mustard gas.

Toxicity of mustard gas-kerateine

We have looked for any toxicity by (*a*) effects upon enzymes, (*b*) effects upon rats and (*c*) tests upon human skin.

(*a*) *Effects upon enzyme systems.* Two were tried, the succinoxidase and the pyruvate oxidase systems. In the former, only slight toxic non-specific effects were noticed, changes not exceeding 15% for amounts of 5–10 mg./3 ml. (Table 3).

Table 3. *The effect of kerateine and mustard gas-kerateine upon the succinoxidase system* (heart muscle)*

Additions	O_2 uptake	Change 50 min. (%)	Difference (%)
	Exp. 1		
Succinate only	309	—	—
+ kerateine (5 mg.)	279	−10	—
+ mustard gas-kerateine (5 mg.)	246	−20	−10
	Exp. 2		
Succinate only	277	—	—
+ kerateine (10 mg.)	248	−10	—
+ mustard gas-kerateine (10 mg.)	210	−24	−14

* We are indebted to Prof. Keilin for this preparation (Keilin & Hartree, 1940) and for cytochrome *c*. We added 0·1 ml. cytochrome *c* for these experiments and used 0·1 ml. succinic dehydrogenase preparation/3 ml. (pH 7·4).

For the pyruvate oxidase system pigeon brain dispersion was used (Peters & Wakelin, 1946) as it was unlikely that the mustard gas-protein product would penetrate a *brei*. The main effect of kerateine and mustard gas-kerateine was a remarkable stabilization of this rather labile enzyme system (see Fig. 2) leading to a total increase in O_2 uptake over the period studied. This is of interest, as it is the first time that we have observed such an action with brain dispersion. It was particularly significant that it was purely an effect in presence of the pyruvate; there was no action on the residual respiration. There is a lower total O_2 uptake for mustard gas-kerateine as compared with kerateine, amounting to −10% in one experiment over a 50 min. period and −15% in another experiment (Fig. 2). This is a small effect, because the 10 mg. could not possibly contain more than 100 μg. mustard gas as a 'half product' which is practically without effect upon this enzyme; it is of a different order of toxicity from that observed for really toxic substances such as the sulphones.

Briefly, these enzyme tests did not reveal any toxicity of the mustard-treated product. The stabilization effect is likely to be due to the SH groups present in the kerateine.

(*b*) *Effects upon the rat.* The compound was fed to a growing rat (96 g.) by replacing 1 g. casein by 1 g. of the compound in each 10 g. of a diet containing 60% rice starch, 20% casein, 5% salt mixture, 2% agar agar, 10% dried yeast, 3% cod-liver oil. A control rat was fed with kerateine in a similar basal diet. Though the rat which was fed the compound did not eat this well and lost weight (13 g. during 5 days), at least 1·0 g. of the product was consumed, equivalent to *c.* 50 mg. of mustard gas (calculated on the S content). The process of mixing the diet was carried out quickly and water reduced to a minimum to minimize possible hydrolysis of the 'half product'.

Dr Short (Prof. Cameron's Department, Experimental Station, Porton) reported (private communi-

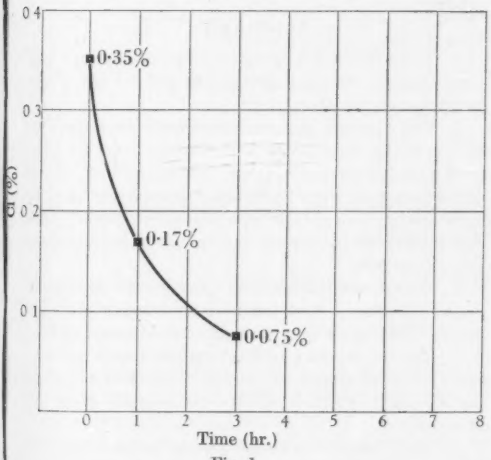

Fig. 1.

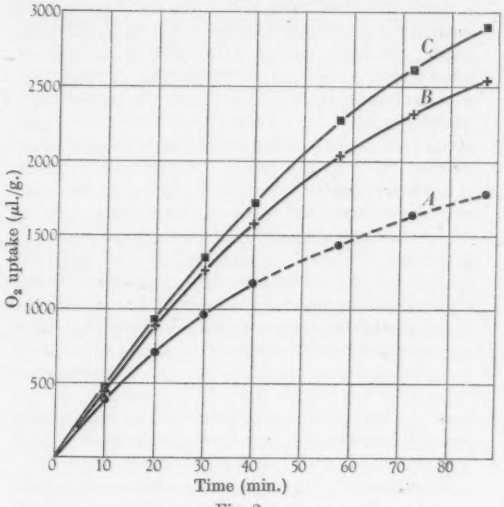

Fig. 2.

Fig. 1. Hydrolysis of mustard gas-kerateine. 2·0 g. samples suspended in 50 ml. water pH 7·4 at 38°. Curve gives percentage Cl in the dried protein.

Fig. 2. Increase in O_2 uptake of pigeon brain 'dispersion' in presence of pyruvate (curve *A*) by addition of mustard gas-kerateine (curve *B*) or kerateine (curve *C*).

cation) that there was no macroscopic or microscopic abnormality in the stomach. The fall in weight was due to low consumption of the diet as a result of the unappetizing character of kerateine, as the control rat also lost weight. Though only one rat was used, the result is regarded as valid evidence of absence of mustard gas toxicity in the dose consumed as even small amounts are detectable histologically. (Subsequent to this work, Kinsey & Grant (1946) fed mustard gas-casein to rats and observed no toxic effect, though the rats lost weight unless the diet was supplemented with methionine and threonine).

Dr Holiday, to whom we are grateful, injected 10 mg. of the compound in saline at pH 7·4 intraperitoneally into a rat without adverse results.

(*c*) *Behaviour in human skin.* The behaviour of these products was peculiar. Tests were done upon two human subjects both reacting with E^+ (raised erythematous patches $3 \times 2{\cdot}5$ cm.) to 1/140 ml. of 1/1000 mustard gas in benzene; in every case 20 mg. of the powder was worn under a patch of cellophan 3 cm. diam., for periods of 2·5 hr. unless otherwise stated.

Kerateine itself was quite non-irritant, applied both in the dry state, even for 24 hr., and as a moist patch. Mustard gas-kerateine was never irritant after recent extraction by stirring for at least 2 hr. at room temperature with acetone. On at least three occasions, however, preparations which had been stood in a desiccator for from 4 to 6 weeks proved irritant (E^+) upon a retest. In the first instance, this was considered to be due to improper extraction and a re-extraction gave a non-irritant powder which did not subsequently become irritant. On a second occasion, a preparation had acquired irritancy (removed by acetone extraction); this was believed at the time to be due to standing (though in a corked tube) in a desiccator, in which were other preparations containing small amounts of mustard gas. On a third occasion, the same preparation (known to have been freed from irritancy (2·5 hr. test) by second extraction with acetone) upon a retest 6 weeks later in which the patch was accidentally worn for 12 hr., gave an E^+ patch, $3{\cdot}8 \times 4$ cm. and again, for 2·5 hr., E, 3×3 cm. Hence we believe that the effect is genuine. Its extent must not be exaggerated; this recovery of irritancy does not correspond to more than 5 μg. as mustard gas in the 20 mg., i.e. 1/4000; but the repetition of the phenomenon is interesting and, if occurring *in vivo*, it would be important.

DISCUSSION

Though some mustard gas combined with the SH, the behaviour of the SH groups in kerateine to mustard gas is different from that found by Stocken & Thompson (1940) for the arsenicals, which attacked the SH groups alone, 75% of which formed rings with As. We think the work provides some explanation of the reason why mustard gas does not appear to abolish the fixed SH reaction in muscle; there is not enough diminution to be noticeable.

It is curious that the mustard gas 'adsorbed' upon kerateine can only be removed from this very fine powder by prolonged extraction with an organic solvent. One might have expected that the step involving solution in ice-cold water would have been adequate to deal with this. Long ago, Dixon & Gooding (1924) drew attention to the permanence of mustard gas adsorbed on silk, etc. and the possibility of adsorbed mustard gas has been considered by Fell & Allsopp (1940–1). The properties of 'adsorbed' mustard gas as distinct from that dissolved in fats needs more study as the substance seems in some way to be stabilized. This might occur through van der Waals's attachment at O atoms, forming a 'pseudo-sulphoxide' structure in which Cl atoms are stable. Whatever is its explanation, it would be a factor in the retention of mustard gas in tissue, available for delayed effects.

This paper embodies the main features of a Report to the Ministry of Supply, no. 39, by Peters in 1941. For an account of the work as a whole, see Peters (1947).

SUMMARY

1. The soluble SH protein product kerateine has been treated with mustard gas at pH 7·4 and room temperature for about 1 hr.

2. The mustard gas combined with the kerateine as shown by the increase in sulphur content. By porphyrindin titration, it was estimated that about one-quarter of the combining mustard gas was in combination with SH groups originally present. This was confirmed by the use of the new silver reaction for thio-ethers.

3. About one-third of the mustard gas had combined to form a half product containing chlorine more slowly hydrolyzable than that of mustard gas.

4. The fine white powder, mustard gas-kerateine, even after thorough extraction with acetone, may show slight irritant effects to human skin on standing.

5. Mustard gas-kerateine was not toxic to the rat; it is not toxic to the succinoxidase system, and stabilizes O_2 uptake of the pyruvate oxidase system, a property also shown by kerateine itself.

This work was carried out as part of a larger programme of research directed by Prof. R. A. Peters for the Ministry of Supply. We are indebted to the Director General of Scientific Research (Defence) for permission to publish it. We are also indebted to Drs Holiday, Ogston, Stocken and Thompson for useful suggestions and to the Physiological Department, Porton, for histological aid. We are also grateful to Prof. Keilin for the succinic dehydrogenase preparation and for the cytochrome *c*.

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The Splitting of some Thio-ether Linkages by Silver Salts under Mild Conditions

By R. A. PETERS and R. W. WAKELIN, *Department of Biochemistry, Oxford*

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In the attempt to decide (Peters & Wakelin, 1941, 1947) whether the compound of mustard gas and kerateine contained thio-ether linked (=C—S—C=) mustard gas, a new reaction was discovered; by suitable treatment at room temperature with Ag or Hg salts and adjusting to an alkaline reaction, some thio-ether linkages which are otherwise stable can be split. This splitting can be shown by a special application of the nitroprusside test.

EXPERIMENTAL AND RESULTS

Nitroprusside reactions

In order to throw light upon the finding that the nitroprusside reaction was reduced in the mustard gas-kerateine compound as compared with kerateine itself, we studied the type of reactions given by nitroprusside with several sulphur compounds. There can be distinguished five types of nitroprusside reactions given by sulphur compounds upon addition of alkali with or without cyanide (0·05 ml. 20 % KCN added to 0·5–1·0 ml. test solution) followed by 0·05 ml. of 5 % sodium nitroprusside (NP). These are listed in Table 1.

To obtain convincing evidence of the splitting of a thio-ether linkage, it is necessary to find a compound which gives no (*b*), (*c*) or (*d*) reaction, but a powerful type (*e*) reaction. The colour must be strong since it is difficult to be certain that a rather weak type (*d*) reaction is not due to disulphide impurities.

Table 1. *Types of nitroprusside reaction*

	Reaction conditions	Colour development	Reagents
(*a*)	Cool	Immediate	Dilute ammonia + NP
(*b*)	Cool	Immediate	Cyanide + NP } (see note 1)
(*c*)	Cool	Delayed	Cyanide + NP }
(*d*)	Warm	Immediate	Cyanide + NP (see note 2)
(*e*)	Cool	Immediate	$AgNO_3$ or $Hg(OAc)_2$ + Cyanide + NP (see note 3)

Note 1. The use of cyanide was described by Walker (1925) for S—S groups, who stated that cystine might take 20 min. to reach maximum colour. If the cyanide is added and the solution allowed to stand for 15–45 min., immediate reaction occurs on addition of nitroprusside; sometimes reduction takes more than 30 min. In contrast, substances like dithiodiglycol react immediately (Type *b*).

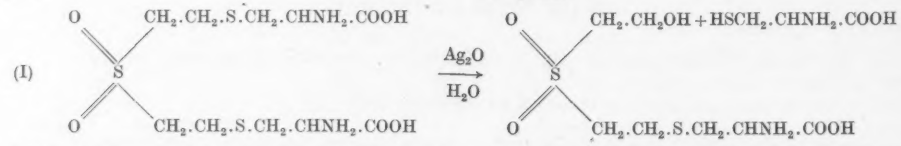
Note 2. This is a variant of (*c*); for differentiation it is better to use 5–10% cyanide. The solution can be warmed to 90° and cooled, or placed in the water-bath at 50° for 10–15 min. (best stoppered).

Note 3. The test solution is treated with $AgNO_3$ or mercuric acetate, brought slowly to pH 7·5–8·0 by 0·1N-NaOH, and shaken for 3–5 min. Cyanide + NP are then added.

To the best of our knowledge (*e*) is a new reaction. It has not yet been thoroughly explored using metals other than Ag and Hg. It is given by cystine, due presumably to the intermediate formation of mercaptide.

Behaviour of some thio-ether compounds

The compound (I) has been found to satisfy the above conditions. It is 2:2-bis-(2′-amino-2′-carboxyethylthio)-diethyl sulphone, obtained by interaction of divinyl sulphone and cysteine, or from mustard gas sulphone (Ford-Moore, 1940). A specimen was kindly given to us by Mr A. H. Ford-Moore.



When an aqueous solution of (I) is treated with cyanide and nitroprusside, there is no magenta reaction; even after warming, this is very slight. When, however, the compound is treated with $AgNO_3$ followed by NaOH to pH 7·8 with shaking, an immediate strong reaction is given, equivalent in intensity to about 50% of its weight of cysteine. Controls showed that a concentration of (I) twenty times that of cysteine does not interfere with a cyanide + NP reaction for cysteine. The reaction occurs equally well *in vacuo*. It may be regarded as a hydrolysis as it involves the addition of water.

The cysteine formed from substance (I) can be isolated as cystine after a silver treatment. The $AgNO_3$ complex with (I) made at slightly acid reaction can be separated at pH 7·0 by centrifuging. After washing and suspending in water and addition of cyanide + nitroprusside, it gives no nitroprusside reaction. If, however, after suspension in water, it is shaken with gradual addition of NaOH to *c.* pH 8·0, it then gives the usual nitroprusside reaction with cyanide; hence in the Ag complex the sulphur linkage is more sensitive to the reaction of OH ions.

That cysteine is reconstituted in this reaction and that the cyanide takes no part in the hydrolysis we have proved as follows: 50–100 mg. of compound (I) are treated in 5 ml. of water with 5 ml. of 10% $AgNO_3$. At first, the addition causes solution; with continued addition a silver complex separates, which is washed after centrifuging. The silver content was 32·5%, slightly over 2 atoms/molecule of compound I. To obtain more complete splitting, the pH must be made more alkaline than that at which the nitroprusside reaction is detectable. The Ag complex is therefore treated with NaOH to pH 8·5, thoroughly shaken and allowed to stand about 5 min. The pH is then adjusted to pH 3·5 with N-HCl and the ppt. is washed with dilute HCl at this pH. The ppt., containing silver and cysteine, is suspended in 3–4 ml. water (pH 3·0) to which are added 50–100 mg. NaI, pH 3·0. Successive treatments with NaI in 3–4 ml. water will eventually liberate cysteine in solution; 0·05–0·1 ml. of each extract is tested, after centrifuging, for cysteine with addition of cyanide + nitroprusside. The first and second extracts may contain no cysteine and may be discarded. (That an actual SH rather than S—S compound is present is proved by tests with nitroprusside and ammonia alone.) The extracts are concentrated to dryness *in vacuo* (35–40°), treated with ammonia and left to oxidize in air. Ammonia is removed *in vacuo*, and the ppt. washed with ice-cold water; after centrifuging, the ppt. is then redissolved in ammonia and the cystine formed by oxidation allowed to crystallize gradually. Hexagonal plates of cystine are obtained by dissolving in acid and reprecipitating by addition of NaOH to pH 3·0; the mother liquor gives the typical slow reaction of Walker (1925) for cystine upon addition of cyanide + NP, and also the Sullivan (1929) reaction. No reaction for H_2S was obtained at any stage with lead acetate paper. After purification the yield of cystine is approx. 10% of the original weight of the compound.

We have similarly isolated cystine from (2-amino-2-carboxyethylthio)-ethylphenyl sulphone

$$C_6H_5SO_2CH_2CH_2SCH_2CH(NH_2)COOH \qquad (II)$$

prepared by Mr Ford-Moore at Porton; the procedure was the same except that we were unable to prepare a satisfactory silver complex (so having to use an excess of $AgNO_3$), and that a more alkaline pH was necessary. We treated 30, 100 and 500 mg. batches with excess of $AgNO_3$ at pH 4·5, followed by addition of 0·1N-NaOH to *c.* pH 10·0. The losses were considerable in purification, which was carried out with some modifications of the procedure described above. Some of the cysteine is lost, presumably at the silver complex stage. In this con-

nexion, it may be noted that Vickery & Leavenworth (1930) state that cystine, when precipitated as the Ag salt and brought to pH 6·0, consists largely of a Ag-cysteine compound giving the colour reaction for cysteine, but in addition, reduction is accompanied by oxidation giving, among other products, ammonia and cysteic acid. Further work upon this reaction is described elsewhere (Ford-Moore, Peters & Wakelin, 1942).

Some applications of the silver reaction for thio-ethers

As previously mentioned it can be shown by the use of this reaction that some thio-ether linked sulphur is present in mustard gas-kerateine (Peters & Wakelin, 1947). Some product formed upon treatment of cysteine with mustard gas also gives the type (*e*) nitroprusside reaction; this has not been isolated, but we presume it to be the half product;* it is left in solution after precipitation of the crystalline dicysteinyl mustard gas product 2:2-bis-(2′-amino-2′-carboxyethylthio)-diethyl sulphide. The latter gives a faint type (*e*) reaction; this was confirmed upon a pure specimen of the bis-compound kindly sent to us by Dr Hellerman.

Preparation of mustard gas-cysteine product. Using Ogston's (1941) modification of the technique of Peters & Walker (1923), excess of mustard gas dissolved in ethanol was added gradually at room temperature to cysteine-HCl (50 mg.) dissolved in 40 ml. water containing a trace of bromothymol blue as indicator; precautions were taken to avoid oxidation by bubbling N_2 through the reaction vessel. NaOH solution (0·2 N) was added at intervals to maintain the pH at *c.* 7·4. On completion of the reaction SH had disappeared (as shown by the absence of type (*a*) reaction). The type (*e*) reaction with Ag salts was strong, giving a colour intensity about 50% of that given by an equal weight of cysteine. Care must be taken to avoid excessive alkalinity as this thio-ether linkage is evidently more easily hydrolyzed by alkali alone than in the case of the sulphone compounds.

Reactions of other thio-ethers. Qualitative reactions with silver salts have been found with other substances containing the thio-ether linkage, though there is much quantitative difference in the splitting. With methionine, rather special treatment is needed. To 0·5 mg. or more of methionine in 0·2 ml. water, 0·05 ml. 10% $AgNO_3$ is added and 0·1 N-NaOH drop by drop with constant shaking as the precipitate separates; cyanide and nitroprusside are then added.

Reactions have been obtained with *S*-carboxymethyl cysteine kindly given by Prof. F. Dickens. The reaction with di*iso*propyl sulphide was slight and very transient. Pure specimens of thiodiglycol give no splitting with silver; impure specimens usually give a type (*c*) nitroprusside reaction because they contain dithiodiglycol as an impurity, as was shown by Peters & Walker (1925); 2:2′-dichlorodiethyl sulphone gives no reaction.

Of several other sulphur compounds tested, for which we are indebted to Dr du Vigneaud, using 0·5 mg. for each test, *dl*-cystathionine and *d*-lanthionine gave faint silver reactions; no reaction was given by *l*-djenkolic acid, *d*-*S*-benzyl homocysteine or *l*-*S*-benzylcysteine.

DISCUSSION

Some thio-ether linked compounds are known to be degraded in varying degree after drastic treatment with alkali (Schönberg & Iskander, 1942); compounds of thiol acids with aldehydes which readily dissociate in alkaline solution have long been known (Schubert, 1935, 1936). The compounds studied here are much more stable. Apart from the chemical interest and possible applications of our silver reaction, we feel that it presents an instance in which the thio-ether linkages can be split at comparatively low temperature near the biological pH. The mechanism of the reactions appears to be the formation of a silver complex, which is more susceptible to splitting by alkali.

This paper embodies the gist of two reports nos. 39 and 53 sent to the Ministry of Supply by Peters in 1941 and 1943.

SUMMARY

1. The compound of divinyl sulphone and cysteine, viz. 2:2-bis-(2′-amino-2′-carboxyethylthio)-diethyl sulphone (made by Ford-Moore) gives no nitroprusside reaction by treatment with cyanide; if, however, it is treated at room temperature with a silver salt at acid pH and the resultant mixture made slightly alkaline, a nitroprusside reaction is given in presence of cyanide. In this new reaction of silver salts it has been proved that cysteine is regenerated from the thio-ether linkage.

2. The reaction has been used as supporting evidence for the presence of some thio-ether linked mustard gas in mustard gas-kerateine.

3. The reactions of some other sulphur compounds have been investigated.

This work was carried out as part of a programme of research for the Ministry of Supply directed by Prof. R. A. Peters. We are grateful to Mr A. H. Ford-Moore, Experimental Station, Porton, and to others mentioned in the text for the provision of compounds. We are grateful also for valuable criticism to members of the team, and to Dr Whittaker for the specimen of monothioethylene glycol. We are indebted to the Director General of Scientific Research (Defence), Ministry of Supply for permission to publish this work.

* After completion of the above work, and after submission of this paper for publication, we had the opportunity of testing the half product mentioned, viz.

$ClCH_2CH_2SCH_2CH_2SCH_2CH(NH_2)CO_2H$,

kindly given by Drs Kinsey and Grant; we can confirm that this compound gives the silver reaction.

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1:2:4-Fluorodinitrobenzene

By H. G. COOK and B. C. SAUNDERS, *University Chemical Laboratory, Cambridge*

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During the war we carried out extensive investigations on the synthesis and physiological properties of organic compounds containing fluorine (McCombie & Saunders, 1941, 1946 *a*, *b*, *c*). In connexion with this work we had occasion to prepare 1:2:4-fluorodinitrobenzene, and as several workers have recently used this compound for other purposes (e.g. Sanger, 1945), its preparation is now given in detail.

1:2:4-Fluorodinitrobenzene was first prepared by Holleman & Beekmann (1904) by nitrating *p*-fluoronitrobenzene which in turn was prepared from *p*-nitroaniline. Later, Gottlieb (1936) obtained it by the action of potassium fluoride on 1:2:4-chlorodinitrobenzene. Neither method is entirely satisfactory. The second method is obviously more direct, but as described by Gottlieb is laborious. It consists in heating 1:2:4-chlorodinitrobenzene dissolved in nitrobenzene with potassium fluoride (2/3 equiv.), cooling, adding more potassium fluoride, reheating, cooling, filtering, and then repeating the whole procedure twice more, thus using 4 equiv. of potassium fluoride. Gottlieb maintains that this procedure is essential, otherwise the potassium fluoride becomes coated with potassium chloride and the reaction stops. We have found, however, that the method can be greatly improved as described in the experimental part. Only 2 equiv. of potassium fluoride are required, and in particular the intermittent additions and filtrations are unnecessary.

Pure 1:2:4-fluorodinitrobenzene is a colourless liquid which darkens slowly on standing, and solidifies when cooled in ice water. In aliphatic compounds containing the C–F link, the fluorine atom is usually less reactive than the chlorine atom in the corresponding chloro compound. In 1:2:4-fluorodinitrobenzene, however, the fluorine atom is more reactive than the chlorine atom in 1:2:4-chlorodinitrobenzene towards many reagents.

Whereas methyl fluoroacetate is a powerful convulsant poison (McCombie & Saunders, 1946*c*), 1:2:4-fluorodinitrobenzene is found to be relatively non-toxic, and does not exhibit any fluoroacetate-like activity. 1:2:4-Fluorodinitrobenzene, however, shows slight vesicant action on human skin, and in this respect it resembles 1:2:4-chlorodinitrobenzene. This vesicant action, though slight, persists with some individuals for a considerable time, therefore the compound should be handled with care.

EXPERIMENTAL

Preparation of 1:2:4-*Fluorodinitrobenzene*

1:2:4-Chlorodinitrobenzene (101 g., 0·5 mol.) was dissolved in dry nitrobenzene (101 g.) and to this solution was added dry, finely powdered KF (60 g., 1 mol.) and the mixture heated to 190–195° for 5 hr., under reflux, with vigorous stirring. The product was then cooled and filtered from inorganic salts, which were washed with hot toluene. The combined toluene filtrates were dried overnight over anhyd. Na_2SO_4. After filtering off the Na_2SO_4, the toluene was distilled off on the water pump and the residue fractionally distilled. The fraction of b.p. 136–138°/2 mm. was collected (yield 66 g., 71 %). This sample was sufficiently pure for most purposes. A second fractionation gave a product which was entirely chlorine free and solidified readily on being cooled in ice water.

The fluorine was estimated by the method of Chapman, Heap & Saunders (1947). Found: F, 10·03; 10·38. Calc. for $C_6H_3O_4N_2F$: F, 10·2 %.

No appreciable reaction took place when NaF, in place of KF, was heated with 1:2:4-chlorodinitrobenzene employing the conditions given above.

Physiological examination

(1) A batch of 23 rabbits, rats, guinea-pigs and mice were exposed to a Ct* of 5000 mg. min./m.^{3} in a 1·87m.^{3} static chamber. The material was sprayed in ether (25 ml.). During exposure the animals suffered from irritant action, but recovered after being removed from the chamber. At the end of a week there were no deaths. The batch was then re-exposed to the same Ct. The irritation seemed more intense and all four guinea-pigs died in 48 hr. The post-mortem examination showed changes typical of those caused by a lung irritant.

(2) Subcutaneous injection into mice, using propylene glycol as solvent: 100 mg./kg. killed 1/6.

(3) *Vesicancy on human skin.* Benzene solutions of 1:2:4-fluorodinitrobenzene (*F*) were dropped on to the right arms of two subjects, and similar solutions of 1:2:4-chlorodinitrobenzene (*Cl*) were dropped on to the left arm. There was free evaporation.

5 mg. drops of 1% solution (i.e. 50 μg.) had no effect within 4 days. 5 mg. drops of 10% solutions (i.e. 500 μg.) produced considerable irritation as follows:

Subject 1. *F patch.* Smarting and swelling in 7 hr., blister without surrounding erythema in 36 hr. No change when blister was pricked on 3rd day. Scar almost healed by 9th day. *Cl patch.* During the first 9 days there was no more than a red weal. By the 12th day there was irritation and swelling which subsided on the 15th day.

Subject 2. *F patch.* Immediate smarting and swelling which persisted for 1 day, and subsided the next day without a blister. After 3 days the skin broke down with formation of pus and scabs. After 8 days only a scar remained. *Cl patch.* Slight redness during first 8 days. Swelling on 9th day and weal rash of vesicles on 10th day. The spots oozed freely on the 14th day. Recovery on 20th day.

* Ct is the dose expressed as concentration mg./m.^{3} × time of exposure (min.).

SUMMARY

1. A convenient method of preparing 1:2:4-fluorodinitrobenzene is described.

2. The compound is relatively non-toxic in comparison with the highly toxic fluoroacetates. It produces, however, a slight vesication on human skin.

Our thanks are due to the Director General of Scientific Research (Defence) for permission to publish the above and to Mr K. J. Carpenter who carried out the physiological examination of the action of the compound on animals.

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The Utilization of Nicotinamide Derivatives and Related Compounds by Mammals, Insects and Bacteria

By P. ELLINGER, G. FRAENKEL and M. M. ABDEL KADER (Member of the Egyptian Ministry of Education Mission), *Lister Institute of Preventive Medicine, London, and the Biological Field Station, Imperial College of Science and Technology, Slough, Bucks*

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This investigation was prompted by the desire to find an antipellagra drug of the efficiency of nicotinamide, but of a greater lasting effect similar to that of nicotindiethylamide (nikethamide) (Ellinger & Coulson, 1944). For this purpose a number of nicotinamide derivatives in which one or both hydrogen atoms of the amide were replaced by alkyl, cyclohexyl, or aryl groups were tested for their ability to form nicotinamide and to exhibit antipellagra activity. This aim was not reached, but the investigation gave results which are of interest, since they give some information about the relation of chemical constitution to pharmacological action and about the ability of widely different species to transform

nicotinamide derivatives and related compounds into nicotinamide or nicotinic acid which are essential to all of them.

Anti black-tongue activity of a large number of such compounds has been studied by Woolley, Strong, Madden & Elvehjem (1938); SubbaRow, Dann & Meilman (1938); Strong, Madden & Elvehjem (1938); SubbaRow & Dann (1938); Waisman, Mickleson, McKibbin & Elvehjem (1940); Smith, Margolis & Margolis (1940) and Teply, Krehl & Elvehjem (1945). These authors found the following to be active: nicotinic acid, nicotinamide, nicotinmethylamide, nicotindiethylamide, nicotinamide glucosidoiodide, nicotinuric acid and to a lesser degree β-picoline, whilst trigonelline, nicotinamide methochloride, pyridine, pyridine-3-sulphonic acid, 6-methylnicotinic acid, 3-acetylpyridine, 3-aminopyridine, quinolinic acid, picolinic acid, *iso*nicotinic acid, hexahydronicotinic acid, nicotinonitrile, pyrazine mono- and 2:3-dicarboxylic acids, and thiazole-5-carboxylic acid were found to be inactive in canine black-tongue. Nicotinuric acid was found inactive by Dann & Handler (1941) and nicotinamide methochloride active by Najjar, Hammond, English, Wooden & Deal (1944).

Antipellagra activity has been mainly studied by Spies and his co-workers (Spies, Bean & Stone, 1938; Spies, Grant & Huff, 1938; Vilter & Spies, 1939; Vilter, Bean & Spies, 1938; Bills, McDonald & Spies, 1939), and by Smith *et al.* (1940). The results are less definite than those obtained for black tongue activity. It is certain, however, that nicotinic acid, nicotinamide, nicotinethylamide and -diethylamide, ethyl nicotinate, quinolinic acid, and pyrazine mono- and 2:3-dicarboxylic acids are active against pellagra. α-Picoline, trigonelline, and 3-aminopyridine are inactive, whilst β-picoline, 2:6-dimethylpyridine-3:5-dicarboxylic acid, and pyridine-3:5-dicarboxylic acid are of doubtful activity.

Nicotinamide methochloride has been found to be formed in the mammalian body after administration of nicotinic acid, nicotinamide or nicotindiethylamide (Najjar & Holt, 1941; Ellinger & Coulson, 1944). The former authors found no increased nicotinamide methochloride elimination after administration of trigonelline, 2:6-dimethylpyridine-3:5-dicarboxylic acid, quinolinic acid, pyridine-3:5-dicarboxylic acid and pyrazine mono- and 2:3-dicarboxylic acids.

Very little is known of the pharmacological and toxicological effects of nicotinamide and related compounds in mammals. The lethal doses of nicotinic acid, nicotinamide, nicotindiethylamide, pyridine, pyridine methochloride, trigonelline, nicotinamide methochloride, and nicotindiethylamide methochloride have been studied for the rat, mouse, and guinea-pig by Chen, Rose & Robbins (1938), Unna (1939) and Brazda & Coulson (1946). Whilst Ackermann (1913) failed to observe any toxic effect of nicotinic acid 'in medium-sized dogs after ingestion of 9 g. during 5 days', Chen *et al.* (1938) report lesions of the intestinal mucosa and central nervous system in dogs after large doses of nicotinic acid.

The need of insects for nicotinic acid seems to be general. This has been demonstrated, e.g. for *Drosophila* (Tatum, 1939, 1941), mosquitoes (SubbaRow & Trager, 1940), *Galleria* (Rubinshtein & Shekun, 1941), *Tribolium* and many other beetles and moths (Fraenkel & Blewett (1943*b*, 1946*a*, *b*), Blewett & Fraenkel, 1944). The effects of nicotinamide and other derivatives of nicotinic acid on insects have, however, never been investigated.

The utilization of a number of compounds related to nicotinic acid by various bacteria (*Staphylococcus aureus*, *Proteus vulgaris*, dysentery bacilli, *Lactobacillus arabinosus*) has been studied by Landy (1938), Fildes (1938), Lwoff (1938), Lwoff & Querido (1939), Dorfman, Koser, Reames, Swingle & Saunders (1939), Pelczar & Porter (1940), Elvehjem & Teply (1943) and v. Euler, Högberg, Karrer, Salomon & Ruckstuhl (1944). Of the compounds tested, nicotinic acid, nicotinamide, methyl, ethyl, propyl and butyl nicotinate, and nicotinuric acid could be utilized by all bacteria examined, nicotinethylamide and -diethylamide and quinolinic acid were reported to be utilized by *Proteus* and dysentery bacilli. β-Picoline, nicotinonitrile, pyridine-3-sulphonic acid only by *Proteus*, tetrahydro-, and hexahydronicotinic acid (nipecotic acid) by *Staphylococcus aureus* and *Proteus*, and picolinic acid and *iso*nicotinic acid by none of the bacteria. Hexahydronicotinic acid had only very little activity in promoting growth of *Lactobacillus arabinosus* (Teply *et al.* 1945). Najjar, Scott & Holt (1943) considered nicotinamide methochloride a powerful growth promoter for *Escherichia coli* and *Haemophilus influenzae*.

In the present investigation a number of new and known nicotinamide derivatives have been examined for their general pharmacological action and toxicity on mice or rats. Their ability to form nicotinamide and nicotinamide methochloride and the speed of elimination was also investigated. Attempts were also made to study the mechanism of the breakdown of the substances in the body. The utilization of nicotinamide derivatives by insects was tested by their effects upon growth and development of larvae of *Tribolium confusum*, and utilization by bacteria by effects on growth and acid formation of *Lactobacillus arabinosus*, *Proteus* and dysentery strains. The formation of nicotinamide methochloride from nicotinamide by *Tenebrio* larvae and by some *Proteus* strains has been investigated.

METHODS

The drugs used in the experiment are shown in Table 1. The purity of hitherto unknown substances made by Bayer was checked by analysis. Hydrochlorides were dissolved in water, bases in equivalent amounts of dilute HCl and acids in equivalent amounts of dilute NaOH. Nikethamide was obtained as 25% aqueous solution.

Experiments on mammals. General pharmacological action and toxicity were observed in mice, hooded rats (Lister stock), and albino rats (own stock of P.E.). The drugs were administered in 5–20% solution by intraperitoneal injections to mice of 20–30 g. and rats of 150–300 g. To find the LD_{50} the region was first explored with doses differing from each other by 50% and, when a suitable

order of dose had been established, groups of 4 mice or rats (2, if material was limited) were given doses differing by 10% from each other; at the same time the general pharmacological effect of the various drugs was studied.

To investigate the increased urinary elimination of nicotinamide methochloride after administration of the various drugs, rats of 200–300 g. were kept in metabolism cages, 24 hr. urine samples were collected and their nicotinamide methochloride content estimated by the method of Coulson, Ellinger & Holden (1944). During this period the rats were on the following diet: 10 g. of a mixture composed of 4·0 g. of a grain mixture, 3·5 g. of extracted liver (British Drug Houses), 1·0 g. of wheat germ, 0·3 g. of dried yeast, 0·5 g. of McCollum's salt mixture, no. 185 (McCollum, Simmonds & Pitz, 1917), 0·6 g. of maize oil and 0·1 g. of cod-liver oil, 13 g. of bread and 1·5 g. of dried milk (Glaxo) in 30 ml. of water. The content of nicotinamide in the diet, estimated chemically (Ellinger & Abdel Kader, 1947), was 1·0 mg. and of nicotinic acid 0·8 mg. A number of 24 hr. urine samples (3–5) were examined for nicotinamide methochloride (N.M.), then nicotinamide (20 mg.) was injected intraperitoneally and the response to this dose examined by determinations continued until the predosing level was regained. From the extra output (extra N.M.) in mg. the response x, was calculated by the following formula:

$$x = \frac{\text{mg. extra N.M.}}{\text{mg. } D \text{ ingested}} \times 100 \times \frac{\text{mol. wt. } D}{\text{mol. wt. N.M.}},$$

where D is the compound ingested. This gives the percentage of the nicotinamide dose which appears in its methylated form in the urine. After an interval of 7 days one of the other drugs was injected in equimolar dose to the rats calibrated with nicotinamide and the response was calculated in a similar manner. All drugs examined were tested in duplicate at least.

In order to gain further information on the transformation of the substituted nicotinamide compounds into nicotinamide methochloride, the following experiments were carried out similarly to those described by Ellinger (1946). Rats were stunned and bled, the various organs were removed, pieces were cut off and weighed in wet state, then cut into slices of 0·2–0·4 mm. thickness. These were suspended in 10 ml. of modified Ringer solution (Krebs & Henseleit, 1932), aerated with an O_2-CO_2 mixture, and 10 μg./ml. of the various substituted nicotinamide derivatives were added. The tubes contained either liver alone, or slices of one of the following organs alone or with liver; kidney, brain, spleen, skeletal muscle, heart muscle, pancreas or intestinal mucous membrane. The mixture was agitated at 37° for 2–3 hr. After cooling to room temperature, the supernatant liquid was separated from the tissue, protein was removed with trichloroacetic acid and by heating to 70–80° followed by centrifuging. Nicotinamide methochloride was estimated in the supernatant liquid by the method of Coulson *et al.* (1944).

Experiments on insects. The experiments were carried out on larvae of *Tribolium confusum* by the method of Fraenkel & Blewett (1943*a*, *b*). The nicotinic acid-free diet consisted of 50 g. of acid-extracted casein (Glaxo), 50 g. of anhydrous glucose, 5 g. of the water-insoluble fraction of yeast (exhaustively extracted with hot water), 1 g. of cholesterol, 2 g. of McCollum's salt mixture no. 185 (McCollum *et al.* 1917) and 10 ml. of water. The following vitamins of the B complex were added (μg./g. dry diet): aneurin, 25; riboflavine, 25; pyridoxin, 12·5; pantothenic acid, 25; choline chloride, 250; inositol, 250. All tests were performed at 25° and 70% relative humidity. The compounds to be tested were added to the diet in various quantities and their effect compared with that of nicotinic acid and nicotinamide. The growth of the larvae, the number of pupae, and the average time of pupation formed the basis for a quantitative comparison.

To study the question whether nicotinamide methochloride is formed by insects, 60 g. of larvae of *Tenebrio* grown in white flour (75% extraction) with the addition of 5% yeast were killed, frozen, ground with sand, dried to about 20 g. weight, extracted with 0·1 N-HCl, and filtered. The filtrate (150 ml.) was dialyzed three times for 2 days against 200 ml. of distilled water each time. The dialysates were combined, reduced to 50 ml. by evaporation *in vacuo* and then examined for nicotinamide methochloride by the method of Coulson *et al.* (1944). 'F_2' was tested for by reactions with 12 N-KOH, $K_3Fe(CN)_6$ and alkaline acetone (Najjar *et al.* 1943).

Experiments on bacteria. The various drugs were tested on the following bacteria which require nicotinic acid or nicotinamide for growth: *Lactobacillus arabinosus*, obtained from Dr T. F. Macrae; *Proteus vulgaris* 401, 3156, 5887, from the National Collection of Type Cultures; *Proteus morganii*, isolated from rat faeces; *Proteus* X 13 *syriac*, from Dr A. Lwoff, Paris; *Shigella sonnei* 2941; Flexner 4832; and Shiga 4837, all from the National Collection of Type Cultures. In addition, the effect of nicotinamide methochloride on growth and acid formation of *Esch. coli* was studied in an ammonium-glucose medium on five strains isolated from human and rat faeces. The medium used for *Lactobacillus arabinosus* was that described by Barton-Wright (1944); for the other bacteria a medium of the following composition was used; K_2HPO_4, 2·25 g.; NH_4Cl, 0·25 g.; $(NH_4)_2SO_4$, 0·25 g.; $FeSO_4.(NH_4)_2SO_4.6H_2O$, 0·01 g.; $MgSO_4.7H_2O$, 0·01 g.; the mixture was made up with distilled water to 250 ml. The pH was adjusted to 7·6 with N-NaOH. The solution was boiled and filtered while hot through a no. 50 filter. The volume was brought to 400 ml. after cooling and the solution was then autoclaved; 0·25 ml. of sterile 0·5 M-glucose solution was added to 8 ml. of the salt solution. After adding the sterile test solutions of the nicotinamide derivatives the volume was made up to 10 ml. with sterile water. Two drops of a thin emulsion of the inoculum were added to the medium. After 2–3 days of incubation at 37°, growth was measured by visual estimation of the turbidity and by determination of the acid produced using 0·1 N-NaOH and bromothymol blue as internal indicator. In each experiment blanks were done without inoculum and without the addition of nicotinamide compounds, and the values of the latter were deducted from the experimental values. Tests with nicotinic acid and nicotinamide at different concentrations were carried out as standards for comparison. The compounds to be tested were used in at least two concentrations. In each case the efficiency of the tested compound in forming acid was compared with that of nicotinic acid.

To examine for possible formation of nicotinamide methochloride from nicotinamide or nicotinic acid, *Proteus vulgaris* 3156 was incubated for 48 hr. both aerobically and anaerobically in a casein hydrolysate medium (Barton-Wright, 1944) containing 1 μg./ml. nicotinamide or nicotinic acid. Samples were autoclaved and examined for

nicotinamide methochloride. The remainder was centrifuged, and the supernatant liquid also examined for nicotinamide methochloride by the method of Coulson *et al.* (1944). Controls were done by incubating the medium alone and by estimating the nicotinamide methochloride content immediately after inoculation without incubation.

RESULTS

Pharmacological action. The pharmacological effect of all the compounds tested was qualitatively almost identical, viz. an action on the central nervous system, excitation with convulsions, narcosis, paralysis of the respiratory and the heat regulation centres. In some cases, nos. 12–16, the body temperature of the animals was little above that of their surroundings and changed with it, if the poisoning lasted for more than 24 hr.; none of these animals recovered. With some of the compounds (1, 4, 6, 7, 18) the exciting action predominated; mice developed a 'morphia tail' and jumped like kangaroos; both mice and rats were very restless, convulsions developed and frequently the animals died as a result of one of these. With most of the other compounds the narcotic action was predominant, the animals fell asleep, respiration deteriorated and after a few convulsions death super-

Table 1. *Toxicity and utilization of nicotinic acid, nicotinamide and related compounds in mice and rats*

(The values given in the column for nicotinamide methochloride response are averages of four experiments; the limiting values are given in brackets.)

		Toxicity (LD50)				Nicotinamide methochloride response*	Period of elimination
		(*a*) Mouse		(*b*) Rat			
No.	Name of compound	(g./kg.)	(mmol./kg.)	(g./kg.)	(mmol./kg.)	(% of theoretical)	(hr.)
1	Nicotinic acid	4·5	36·55	3·75	30·90	11·8 (7·9–13·3)	48
4	Nicotinamide	1·75	14·32	1·50	12·28	22·7 (18·2–27·8)	48
5	Nicotinethylamide†	0·65	4·34	0·52	3·47	17·2 (14·3–19·1)	48
6	Nicotindiethylamide	0·42	2·36	0·45	2·55	16·8 (14·7–18·2)	72
7	Nicotinallylamide	0·55	3·40	0·45	2·78	15·7 (13·6–16·9)	48
8	Nicotin*cyclo*hexylamide	0·35	1·71	0·45	1·99	0	—
9	Nicotin-(2′-methyl*cyclo*hexyl)-amide	0·175	0·80	0·20	0·91	0	—
10	Nicotin-(3′-methyl*cyclo*hexyl)-amide	0·12	0·51	0·13	0·60	0	—
11	Nicotin-(4′-methyl*cyclo*hexyl)-amide	0·18	0·83	0·20	0·91	0	—
12	Nicotinphenylamide	0·375	1·90	0·40	2·02	6·5 (4·7–8·3)	72
13	Nicotindiphenylamide	0·07	0·26	0·05	0·18	0	—
14	Nicotin-(4′-methoxyphenyl)-amide	0·35	1·53	0·40	1·76	6·2 (3·8–7·6)	72
15	Nicotinbenzylamide	0·08	0·38	0·07	0·34	5·8 (4·2–6·9)	72
16	Nicotindibenzylamide	0·035	0·12	0·035	0·12	0	—
17	Trigonelline hydrochloride	4·80	27·68	5·20	30·00	0	—
18	Nicotinamide methochloride	0·48	2·78	0·95	5·50	62·7 (56·2–68·3)	24
19	Nicotinonitrile	0·15	1·44	0·20	1·92	0	—
23	Quinolinic acid	0·45	2·69	0·47	2·81	5·2 (3·7–5·9)	48
24	2-Carboxypyridine-3-carboxylic acid *cyclo*hexylamide	0·35	1·41	0·35	1·41	0	—
29	β-Picoline	0·45	4·85	0·48	5·16	41·3 (35·7–53·4)	48–72

* See p. 561.

† The prefix "nicotin-" is used in this paper for the radical

Nicotinethylamide, therefore, stands for the compound

Table 2. *Utilization of nicotinic acid, nicotinamide and related compounds by* Tribolium

No.	Name of compound	Amount of drug in diet (μg./g.)	Surviving larvae		No. of pupae	Average time of pupation (days)
			No.	Size (mm.)		
1	Nicotinic acid	2	—	—	14	44·6
		4	—	—	16	37·4
		8	—	—	20	37·6
		16	—	—	20	35·8
		32	—	—	18	35·4
2	Methyl nicotinate		Same activity as nicotinic acid			
3	Ethyl nicotinate	8	—	—	19	45·0
		16	—	—	18	36·1
		32	—	—	20	35·8
		64	—	—	18	35·6
4	Nicotinamide	2	20	3–5	0	0
		4	—	—	18	41·1
		8	—	—	17	37·0
		16	—	—	19	35·4
		32	—	—	19	36·1
5	Nicotinethylamide	100	19	3–5	0	0
		300	—	—	20	49·8
		900	—	—	15	35·8
6	Nicotindiethylamide	5	5	2–3	0	0
		25	12	2–3	0	0
		125	16	2–3	0	0
		625	—	—	19	40·4
7	Nicotinallylamide	20	17	4–5	0	0
		40	—	—	20	39·5
		80	—	—	17	34·3
8	Nicotin*cyclo*hexylamide	1000	—	—	Few	>100
9	Nicotin-(2′-methyl*cyclo*hexyl)-amide	1000	—	—	Few	>100
11	Nicotin-(4′-methyl*cyclo*hexyl)-amide	1000	—	—	Few	>100
12	Nicotinphenylamide	100	—	—	18	35·5
		300	—	—	19	37·3
		900	—	—	18	38·0
14	Nicotin-(4′-methoxyphenyl)-amide	20	—	—	17	37·1
		40	—	—	19	36·1
		80	—	—	13	35·3
15	Nicotinbenzylamide	100	19	3–4	0	0
		1000	—	—	17	36·7
18	Nicotinamide methochloride	100	No growth		0	0
		500	No growth		0	0
		2500	—	—	Few	>100
19	Nicotinonitrile	100	—	—	Few	>100
		1000	—	—	Few	>100
23	Quinolinic acid	20	No growth		0	0
		100	No growth		0	0
		500	20	4	0	0
		2500	—	—	17	39·0
29	β-Picoline	250	17	4	0	0
		2500	—	—	16	60
		5000	—	—	19	49
		10000	—	—	15	44
		20000	—	—	18	40

37-2

vened from paralysis of the respiratory centre. Some of the compounds (nos. 1, 7 and 13) produced a peripheral vasodilatation, another (no. 29) a distinct hypersensitivity to touch of tail and legs. Generally, death occurred within 2 hr. of the injection of the compound. If this critical period was overcome there was generally complete recovery. In a few cases, however, an apparent recovery was followed after 24–48 hr. by collapse and death from respiratory paralysis. In nearly all the compounds, the LD50 was very well defined and frequently one quarter or one half of the lethal dose had no recognizable effect on the animals.

Nicotinamide methochloride formation by animal tissues in vitro. The experiments carried out with tissue slices to identify the organ responsible for the breakdown of the drug to nicotinamide or nicotinic acid gave negative results. Nicotinamide methochloride was produced only, when either nicotinamide was shaken with liver slices, or nicotinic acid with liver and kidney, or liver and brain slices.

The utilization of various pyridine derivatives by insects

The following compounds produced neither growth of larvae nor pupation in doses up to 1 mg./g. dried food: nicotin-(3′-methyl*cyclo*hexyl)-amide, nicotindiphenylamide, nicotindibenzylamide, trigonelline hydrochloride (prepared according to Hantzsch, 1886), 6-hydroxynicotinic acid (Light and Co.), picolinic acid (Roche), pyridine-4-carboxylic acid (Light and Co.), 2-carboxypyridine-3-carboxylic acid *cyclo*hexylamide (Bayer), pyridine-3:4-dicarboxylic acid (Light and Co.), pyridine-2:5-dicarboxylic acid (Roche), pyridine, α-picoline (B.D.H.) and γ-picoline (Light and Co.).

There was no growth at all when the larvae were fed the basal diet. Usually the minimum concentration of nicotinic acid in the diet required for optimal growth was 8 μg./g., but on one occasion optimal growth was obtained with 4 μg./g. In all experiments the efficiency of methyl nicotinate equalled that of nicotinic acid, whilst the ethyl ester had about one quarter and nicotinamide about one half of the activity of nicotinic acid.

Nicotinamide methochloride content of Tenebrio *larvae.* An extract obtained by the addition of dilute HCl (450 ml.) to *Tenebrio* larvae (60 g.) contained 7·4 μg. nicotinamide methochloride/60 g. wet or 20 g. dry larvae. The alkaline *iso*butanol solutions of the Coulson, Ellinger & Holden test gave a greenish fluorescence with 12 N-NaOH which faded on standing at room temperature, turning yellow. With dilute ferricyanide a deep violet fluorescence developed and the addition of alkali and acetone produced a yellow colour with green fluorescence. This proved the identity of the fluorescent substance with 'F_2'.

The utilization of various pyridine derivatives by bacteria

Compounds no. (7) nicotinallylamide (Bayer), (8) nicotin*cyclo*hexylamide (Bayer), (9) nicotin-(2′-methyl*cyclo*hexyl)-amide (Bayer), (10) nicotin-(3′-methyl*cyclo*hexyl)-amide (Bayer), (11) nicotin-(4′-methyl*cyclo*hexyl)-amide (Bayer), (12) nicotinphenylamide (Bayer), (13) nicotindiphenylamide (Bayer), (14) nicotin-(4′-methoxyphenyl)-amide (Bayer), (15) nicotinbenzylamide (Bayer), (16) nicotindibenzylamide (Bayer), (17) trigonelline hydrochloride (Hantzsch, 1886), (18) nicotinamide methochloride (Karrer, Schwarzenbach, Benz & Solmssen, 1936) (Roche), (19) nicotinonitrile (Dr J. Walker, National Institute for Medical Research) and (24) 2-carboxypyridine-3-carboxylic acid *cyclo*hexylamide (Bayer) were tested. They produced no growth, but a small amount of acid, generally less than 1/100th of that produced with nicotinic acid by *Lactobacillus arabinosus* and neither growth occurred nor acid was produced by any of the other bacteria tested when the various drugs were applied in doses up to 0·03 or 0·05 μg./ml.

The effects of nicotinic acid and nicotinamide on the growth of *Proteus vulgaris* 5887, *Shigella dysenteriae* (Shiga) 4837 and Flexner 4832 were so small that tests of the related compounds on these bacteria were abandoned. No increased growth or acid formation resulted from addition of nicotinamide methochloride to cultures of *Esch. coli* in doses of 0·0001–10 μg./ml., but a distinct inhibition was observed which gradually increased with rising concentration.

The amounts of nicotinamide methochloride formed when *Proteus vulgaris* 3156 was incubated with nicotinamide or nicotinic acid in Barton-Wright medium for 48 hr. are shown in Table 4. No nicotinamide methochloride was found in culture-free medium after 48 hr. incubation or in the unincubated inoculum. Thus no nicotinamide methochloride was released from intact cells into the medium. More nicotinamide methochloride was formed from nicotinamide than from nicotinic acid and an increase in concentration of the substrate from 0·1 μg./ml. to 1·0 μg./ml. did not significantly increase the formation of nicotinamide methochloride. Nicotinamide methochloride formation from nicotinic acid and nicotinamide was greater under anaerobic than under aerobic conditions. The tests of the fluorescent alkaline *iso*butanol solutions with 12 N-NaOH, ferricyanide and alkaline acetone indicated the presence of 'F_2'.

DISCUSSION

Toxicity. The general pharmacological effects on mice and rats, namely excitement and narcosis of the central nervous system and particularly of the

Table 3. *Utilization of nicotinic acid, nicotinamide and related compounds by various bacteria*

No.	Name of compound	Drug content of medium (μg./ml.)	Growth (arbitrary units)	Acid formed (ml. 0·1 N-NaOH)	Efficiency compared with that of nicotinic acid	Drug content of medium (μg./ml.)	Growth (arbitrary units)	Acid formed (ml. 0·1 N-NaOH)	Efficiency compared with that of nicotinic acid
		Lactobacillus arabinosus				*Proteus vulgaris* 401			
1	Nicotinic acid	0·005	+	0·67		0·0025	+	0·16	
		0·01	+ +	1·37		0·005	+ +	0·21	1
		0·02	+ + +	2·69	1	0·01	+ +	0·26	
		0·04	5+	4·46		0·02	+ + +	0·31	
		0·08	6+	4·91					
		0·12	8+	5·99					
4	Nicotinamide	0·005	+	0·70		0·0025	+	0·15	
		0·01	+ +	1·37	1	0·005	+ +	0·19	1
		0·02	+ + +	2·73		0·01	+ +	0·25	
		0·04	5+	3·86	0·86	0·02	+ + +	0·31	
		0·08	6+	4·37	0·89				
		0·12	8+	5·48	0·92				
5	Nicotin-ethylamide	0·08	0	0·045		0·02	±	0·07	0·2
		0·12	0	0·065	0·01	0·03	+	0·09	
		0·20	0	0·155					
6	Nicotin-diethylamide	0·08	0	0·045		0·02	0	0	0
		0·12	0	0·045	<0·01	0·03	0	0	
		0·20	0	0·090					
23	Quinolinic acid	0·08	±	0·14		0·02	+	0·06	0·2
		0·12	±	0·19	*c.* 0·012	0·03	+	0·07	
		0·20	+	0·33					
29	β-Picoline	0·08	±	0·03		0·02	+	0·06	0·2
		0·12	±	0·11	*c.* 0·008	0·03	+	0·07	
		0·20	+	0·24					
		Proteus X 13				*Proteus morganii*			
1	Nicotinic acid	0·0025	+ + +	0·18		0·0025	+	0·11	
		0·005	+ + + +	0·31	1	0·005	+ +	0·45	
		0·01	6+	0·41		0·01	+ + +	0·80	1
		0·02	8+	0·52		0·02	+ + + +	1·79	
						0·04	6+	2·12	
						0·06	8+	2·24	
4	Nicotinamide	0·0025	+ +	0·11	0·67	0·0025	+	0·15	
		0·005	+ + + +	0·23	0·74 0·79	0·005	+ +	0·38	
		0·01	6+	0·39	0·95	0·01	+ + +	0·79	1
		0·02	8+	0·45	0·87	0·02	+ + + +	1·75	
						0·04	6+	2·23	
						0·06	8+	2·26	
5	Nicotin-ethylamide	0·05	±	0·05	0·02	0·05	0	0	0
		0·1	+	0·09					
6	Nicotin-diethylamide	0·05	±	0·05	0·02	0·05	0	0	0
		0·1	±	0	0				
23	Quinolinic acid	0·02	+	0·17	0·12	0·02	+	0·40	0·15–0·25
		0·03	+	0·21		0·03	+	0·41	
29	β-Picoline	0·02	+	0·15	0·1	0·02	+	0·37	0·12–0·16
		0·03	+	0·20		0·03	+	0·40	

Table 4. *Nicotinamide methochloride formation from nicotinamide and nicotinic acid by* Proteus vulgaris 3156 *after aerobic and anaerobic incubation at 37° for 48 hr.*

Substrate	Concentration of substrate (μg./ml.)	Nicotinamide methochloride formed (μg./10 ml.) on (a) Aerobic incubation: In medium	(a) Aerobic incubation: In organisms	(b) Anaerobic incubation: In medium	(b) Anaerobic incubation: In organisms
Nicotinamide	0·1	0	1·71	0	2·18
	1·0	0	1·71	0	2·12
Nicotinic acid	0·1	0	0·62	0	0·82
	1·0	0	0·75	0	1·02

respiratory centre, seem to be quite separate from the metabolic action of nicotinamide. They occur immediately after the injection and disappear after a few minutes, when sublethal doses are given. It is almost certain that in consequence of this action on the central nervous system, no single dose can be administered which can seriously diminish the methyl donors of the body. The toxicity observed in these experiments is similar to that reported by Chen *et al.* (1938), Unna (1939) and Brazda & Coulson (1946) for nicotinic acid, nicotinamide, nikethamide, and trigonelline; but there is a discrepancy as to the relative toxicity of nicotinamide and its methochloride. Brazda & Coulson found the latter to be less toxic than nicotinamide (by 33%), while our results indicate that nicotinamide methochloride is twice as toxic as nicotinamide itself; this finding is not in accordance with the generally accepted idea that this methylation is a detoxication (Perlzweig & Huff, 1945). The toxicity of each of the compounds examined is almost identical for mice and rats with the exception of nicotinamide methochloride which is about twice as toxic for the mouse as for the rat. The toxicity of the various compounds examined rises continuously from the fairly hydrotropic nicotinic acid, trigonelline, nicotinamide and nicotinamide methochloride by more than 100 times to the lipotropic nicotin-diphenyl- and -dibenzyl-amides. Since the main point of attack is presumably on the lipids of the central nervous system, the increased toxicity of the phenyl and benzyl compounds might be related to the partition coefficients between aqueous and lipid phases. This factor is likely to favour concentration of the more toxic compounds in lipids.

Conversion to nicotinic acid in mammals. The ability to increase the formation and elimination of nicotinamide methochloride is based on the intermediary formation of nicotinamide. Antiblack-tongue and antipellagra activities are certainly a property of nicotinamide. Therefore one may attribute antiblack-tongue and antipellagra activities to those compounds which give rise to an intermediary formation of nicotinamide. There is general agreement among the various investigators mentioned in the introduction, that nicotinamide as well as nicotinic acid, its salts and esters and nicotinuric acid possess antiblack-tongue and antipellagra activities. Our experiments have shown a nicotinamide methochloride response to all of these substances which were examined. If the activities against black-tongue and against pellagra respectively are identical, then the results with regard to quinolinic acid and nicotinamide methochloride are difficult to explain. Woolley *et al.* (1938) did not find any antiblack-tongue activity with quinolinic acid which seemed to have a marked antipellagra effect in the experiments of Vilter & Spies (1939). Our experiments are in agreement with the findings of the latter workers. Nicotinamide methochloride was found to be inactive against canine black-tongue by Woolley *et al.* (1938), and later by Teply *et al.* (1945), but was claimed to be active by Najjar *et al.* (1944). The experiments of Elvehjem and co-workers are, however, not fully conclusive because the compound was given orally and with this route of administration only a small proportion is absorbed from the intestine (Ellinger & Coulson, 1944; Ellinger, 1947). Najjar's work needs confirmation and our results are not able to decide the matter in question. β-Picoline has antiblack-tongue activity, but to a much lower degree than the other compounds examined and found active by Woolley *et al.* (1938). In our experiments the response to β-picoline was greater than to all other compounds tested except nicotinamide methochloride. It is difficult to explain this fact in the present state of our knowledge of the mechanism of the intermediary process. Unfortunately the method used by Perlzweig, Bernheim & Bernheim (1943) and Ellinger (1946) to elucidate the mechanism of methylation of nicotinamide and of the formation of nicotinamide from nicotinic acid yielded no results. We do not know definitely, whether the substituted nicotinamide derivatives are broken down to nicotinic acid or nicotinamide. However, the quantitative nicotinamide methochloride response to the various compounds yields some information. The response by the rat to extradietary doses is 22·7% to nicotinamide and 11·8% to nicotinic acid, similar to the values found in man by Ellinger & Coulson (1944). A breakdown to nicotinic acid and intermediary formation of nicotinamide and nicotinamide methochloride would be limited by the nicotinic acid response to values below 11·8%; the alkyl derivatives, mono- and di-ethyl- and monoallyl-amides show, however, responses of 17·2, 16·8 and 15·7% respectively, which are intermediate between those to nicotinamide and nicotinic acid. For these three compounds dealkylation to the amide is most probable; that such a reaction can occur follows from the observations of Elson, Goulden & Warren (1946) who found that dimethylaniline is partly eliminated as *p*-aminophenylsulphonic acid by the rat.

The monoaryl derivatives of nicotinamide (nos. 12, 14 and 15) too produced an increase of nicotinamide methochloride elimination, the response being about 6%. This low value might be due to the fact that these monoaryl derivatives break down to nicotinic acid and not to nicotinamide. The diaryl derivatives (nos. 13 and 16) and the *cyclo*hexyl derivatives (nos. 8, 9, 10, 11 and 24) and nicotinonitrile (no. 19) do not appear in the urine as nicotinamide methochloride, and it is concluded that their breakdown does not lead to either nicotinamide or nicotinic acid. Quinolinic acid is decarboxylated in the 2-position and about 5% of it appears in the

urine as nicotinamide methochloride, a response similar to that of the monoaryl derivatives. From trigonelline no nicotinamide methochloride is formed. On the average 62·7 % of the administered nicotinamide methochloride is eliminated in the urine; similar values were observed in man after parenteral ingestion (Ellinger & Coulson, 1944; Perlzweig & Huff, 1945). The remainder is probably eliminated with the bile and destroyed by the intestinal flora (Ellinger, 1947). There remains only β-picoline (no. 29) which appears in the urine as nicotinamide methochloride to the extent of 35·7–53·4 %, a response much higher than with nicotinamide. This, together with its low antiblack-tongue and antipellagra activity, can only be understood, if it is assumed that its metabolic transformation into nicotinamide methochloride occurs in such a manner that nicotinamide is not involved as an intermediate; it may be suggested that methylation in the 1-position precedes oxidation of the 3-methyl group.

Conversion to nicotinic acid by insects. While in the rat, besides nicotinic acid and nicotinamide, the alkyl and monoaryl derivatives were comparatively well utilized, *Tribolium* was found to be more exacting. Nicotinic acid, its esters, and nicotinamide were well utilized, but most of the other compounds tested had little activity. The only exceptions were nicotinallylamide (no. 7), nicotin-(4′-methoxyphenyl)-amide (no. 14) and nicotinphenylamide (no. 12), whose activity was about 10–20 % of that of nicotinic acid. A definite, but much lower activity was shown by nicotinmono- and -di-ethylamides (nos. 5 and 6), nicotinbenzylamide (no. 15), quinolinic acid (no. 23) and β-picoline (no. 29). This activity might be due to a slight contamination of about 2 % with nicotinamide or nicotinic acid from which all the compounds tested except β-picoline and quinolinic acid are made. This is unlikely since most of these substances showed no activity in bacteria. It appears therefore, that all these substances are utilized by *Tribolium*, if only to a small extent.

Utilization of the various pyridine derivatives by bacteria. In most of the bacteria examined nicotinamide and nicotinic acid were utilized almost equally well except for *Proteus* X 13 which utilized the former by about 20 % less well than the latter. All the other compounds were utilized, if at all, to a much lower degree. *Lactobacillus arabinosus* utilized only nicotinic acid and its amide, in agreement with the results of Elvehjem & Teply (1943), quinolinic acid, β-picoline and nicotinonitrile. The small effect of other derivatives might be explained by contamination with one of the two active compounds. In *Proteus vulgaris* 401 and 3156, nicotinethylamide (no. 5) quinolinic acid (no. 23) and β-picoline (no. 29) showed an activity of about 20% of that of nicotinic acid. In *Proteus* X 13 these compounds and nicotindiethylamide (no. 6) showed an even smaller activity which, in the case of nicotinmono- and -di-ethylamides, can be attributed to a slight contamination with nicotinamide or nicotinic acid. The slight effect of these compounds is in general agreement with the findings of Lwoff & Querido (1939). The positive results reported by the French workers for nicotinonitrile may be due to impurities. *Shigella sonnei* utilized, of all compounds tested, only nicotinic acid and its amide and, to a much smaller extent, quinolinic acid and β-picoline. This confirms the findings by Dorfman *et al.* (1939) also with regard to nicotinonitrile. The positive effect found by these workers for nicotindiethylamide can be attributed to contamination with nicotinic acid or amide. The findings of Najjar *et al.* (1943) that nicotinamide methochloride promotes the growth of *Esch. coli* could not be confirmed. Najjar's results may also have been caused by heavy contamination with nicotinamide or nicotinic acid. The antiblack-tongue effect of nicotinamide methochloride (Najjar *et al.* 1944) can be explained in a similar manner. Nicotinic acid and nicotinamide promote growth with *Esch. coli* (Ellinger & Abdel Kader, 1947).

General conclusion. Comparing the utilization by rat, insect and bacterium of the different compounds tested one realizes that different mechanisms play a role in the transformation of the various compounds into either nicotinamide or nicotinic acid. The rat is able to convert most of the compounds, all alkyl and monoaryl derivatives of nicotinamide and quinolinic acid and β-picoline, into either nicotinamide or nicotinic acid. The mechanisms involved consist in either dealkylation, deamination, decarboxylation or oxidation. The two latter processes seem to occur in insects and in those bacteria tested, whilst dealkylation and deamination are much more restricted in insects and almost entirely absent in bacteria.

SUMMARY

1. The general pharmacological action and toxicity of a number of substituted nicotinamide derivatives and of a few other related compounds have been examined in the mouse and rat. Their conversion of these compounds into nicotinamide methochloride by the rat has also been tested. Relations between physico-chemical properties and toxicity and between chemical constitution and metabolic transformation into nicotinamide methochloride have been discussed.

2. This utilization by the rat has been compared with that by insects and bacteria. It was found that all those compounds which could be utilized by bacteria were also utilized by the higher species, similarly all the substances which were utilized by insects were utilized by rats, but not vice versa.

3. In addition to nicotinamide and nicotinic acid, rats utilized all alkyl and monoaryl nicotinamides, quinolinic acid and β-picoline, but not dialkyl or *cyclo*hexyl nicotinamides or nicotinonitrile. Insects utilized nicotinamide and nicotinic acid and their esters, and less well the mono- and di-alkyl and monoaryl nicotinamides, and still less quinolinic acid and β-picoline. Of the bacteria examined, all utilized nicotinic acid and nicotinamide, quinolinic acid and β-picoline, but only *Proteus vulgaris* and *Proteus* X13 utilized the monoethylamide; the latter also utilized the diethylamide and *Lactobacillus arabinosus* also nicotinonitrile.

4. Nicotinamide methochloride was found to be present in *Tenebrio* larvae and was formed by *Proteus vulgaris* from nicotinic acid and nicotinamide.

The authors are deeply indebted to the late Dr H. Schütze for advice with the bacteriological work; they wish to thank Dr A. Emmanuelowa for isolation of *Esch. coli* from faeces and for maintaining the various cultures used, Miss M. Blewett, Miss J. Vaughan Morgan and Mr W. Wolff for technical assistance, Sir P. Hartley for supply of aneurin, riboflavin, pyridoxin, calcium pantothenate and biotin, Dr T. F. Macrae for the supply of vitamin-free casein hydrolysate, cystine, adenine, guanine, xanthine, uracil, *dl*-tryptophan and of a strain of *Lactobacillus arabinosus*, Dr A. Lwoff, Paris for a culture of *Proteus* X 13, Dr J. Walker for a sample of nicotinonitrile, Bayer Products Ltd. for the supply of the substituted nicotinamide derivatives, and Roche Products Ltd., for nicotinamide and nicotinic acid. One of the authors (G. F.) is indebted to the Medical Research Council for a grant for the study of insect nutrition, and the other (P.E.) wishes to acknowledge a grant from the Ella Sachs Plotz Foundation, Boston, Mass., for technical assistance.

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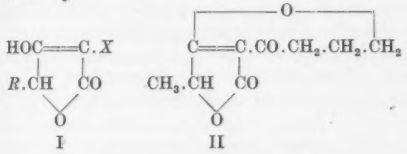
Studies in the Biochemistry of Micro-organisms

75. DEHYDROCAROLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM CINERASCENS* BIOURGE

By A. BRACKEN and H. RAISTRICK
Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(*Received* 19 *March* 1947)

A series of acidic mould metabolic products, each of which is a derivative of tetronic acid (I, $R=H$; $X=H$) has been reported previously from this laboratory:

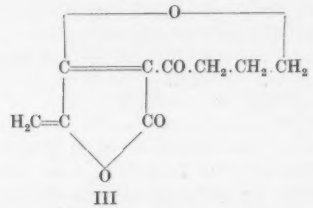


I II

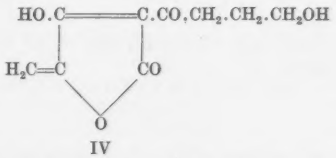
These products are: (*a*) *l*-γ-Methyltetronic acid. [I, $R=$—CH_3; $X=H$. Clutterbuck, Raistrick & Reuter, 1935*c*.] (*b*) Carolic acid. [II; hydrated form, I, $R=$—CH_3; $X=$—$CO.CH_2.CH_2.CH_2OH$. Clutterbuck, Haworth, Raistrick, Smith & Stacey, 1934; Clutterbuck *et al.* 1935*a*.] (*c*) Carolinic acid. [I, $R=$—CH_3; $X=$—$CO.CH_2.CH_2.COOH$. Clutterbuck *et al.* 1934, 1935*a*.] (*d*) Carlic acid. [I, hydrated form, $R=$—$CH_2.COOH$; $X=$—$CO.CH_2.CH_2.CH_2OH$. Clutterbuck *et al.* 1934, 1935*b*.] (*e*) Carlosic acid. [I, $R=$—$CH_2.COOH$; $X=$—$CO.CH_2.CH_2.CH_3$. Clutterbuck *et al.* 1934, 1935*b*.] (*f*) Terrestric acid. [I, hydrated form, $R=$—CH_3; $X=$—$CO.CH_2.CH_2.CHOH.CH_2.CH_3$. Birkinshaw & Raistrick, 1936.]

Products *a*, *b*, *c*, *d* and *e* were isolated from culture filtrates of *Penicillium Charlesii* G. Smith, and product *f*, terrestric acid, from culture filtrates of *P. terrestre* Jensen.

The present communication deals with a hitherto undescribed member of this series which was isolated from culture filtrates of a strain of *P. cinerascens* Biourge and for which the name *dehydrocarolic acid* is proposed. This substance has been shown to have the structural formula III or, in the hydrated form, IV.



III



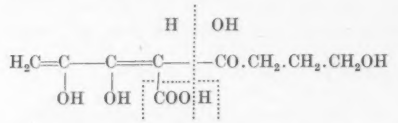
IV

Hence dehydrocarolic acid hydrate is α-(*γ-hydroxy-n-butyryl*)-*γ-methylenetetronic acid*.

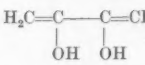
The experimental evidence on which this structural formula is based is as follows: (1) Dehydrocarolic acid, $C_9H_8O_4$, is a colourless, crystalline, monobasic acid. (2) Like carolic acid its aqueous solution turns Congo red paper a deep blue colour, and gives the same characteristic bright orange colour with ferric chloride. (3) It gives finally the same deep bluish-red colour on warming with concentrated sulphuric acid as does carolic acid. (4) Like carolic acid it is readily soluble in chloroform, but unlike this acid, which in aqueous solution is strongly dextrorotatory, dehydrocarolic acid is optically inactive. (5) On hydrolysis with boiling 2N-sulphuric acid some polymerization takes place and carbon dioxide, diacetyl and butyrolactone are formed though not in a theoretical yield. No polymerization is evident when carolic acid is hydrolyzed under the same conditions and the products of hydrolysis are one molecule each of carbon dioxide, acetoin and butyrolactone. (6) On catalytic reduction in hydrogen with a palladium-charcoal catalyst 2 atoms of hydrogen are absorbed and good yields of crystalline dl-*carolic acid*, $C_9H_{10}O_4$, are obtained. This substance, on acid hydrolysis, gives one molecule each of carbon dioxide, acetoin and butyrolactone (cf. dehydrocarolic acid). (7) On treatment with ozonized oxygen, formaldehyde is formed and was isolated as the dimedone derivative in a yield of 64 % of the theoretical for the formation of one molecule. Hence it is clear that dehydrocarolic acid contains the $CH_2{=}C$ grouping.

The structural formulae, III and IV, explain satisfactorily the following experimentally established facts. (1) From its general chemical properties dehydrocarolic acid is clearly a derivative of tetronic

acid. (2) The optical inactivity of dehydrocarolic acid, because of the absence of an asymmetric carbon atom, in contrast to the optical activity of all the other tetronic acid derivatives listed. (3) The products of acid hydrolysis are carbon dioxide, diacetyl and butyrolactone. Thus if the rings are opened, structures III and IV become:



and this structure on breakdown, would give the di-enol form,



of diacetyl, carbon dioxide, and, initially, γ-hydroxybutyric acid. (4) The ready conversion of dehydrocarolic acid to *dl*-carolic acid on catalytic reduction through the change of $CH_2{=}C$ to $CH_3.C$. (5) The presence of the terminal $CH_2{=}C$ grouping and because of this grouping the tendency of dehydrocarolic acid to polymerize.

In addition to dehydrocarolic acid smaller amounts of carlosic acid, product *e* (p. 569) were isolated.

The culture filtrate of this strain of *Penicillium cinerascens* grown on Czapek-Dox solution completely inhibited the growth of *Staphylococcus aureus* at a dilution of 1:80. This was satisfactorily explained by the isolation of the antibiotics, gliotoxin (Weindling & Emerson, 1936; Johnson, Bruce & Dutcher, 1943; Johnson, McCrone & Bruce, 1944) and spinulosin (Birkinshaw & Raistrick, 1931; Oxford and Raistrick, 1942).

EXPERIMENTAL

Culture and cultural conditions

The culture used throughout this investigation was isolated in 1943 from Cumberland soil by Mrs J. M. Webb and was identified by Mr George Smith as a strain of *Penicillium cinerascens* Biourge. It bears the London School of Hygiene and Tropical Medicine (L.S.H.T.M.) catalogue number M. 538.

The culture medium used was Czapek-Dox solution of the following composition: glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water, to 1 l. The medium was distributed in 350 ml. amounts in a number of 1 l. conical flasks plugged with cotton wool, sterilized, inoculated with a spore and mycelium suspension of *P. cinerascens* and incubated at 24°.

ISOLATION OF GLIOTOXIN AND SPINULOSIN

Individual flasks were harvested from time to time and tested for antibacterial activity against the L.S.H.T.M. strain of *Staphylococcus aureus*, employing the serial dilution method described by Clutterbuck, Lovell & Raistrick (1932) using 2% glucose heart broth, and recording the results obtained after 24 hr. incubation at 37°.

The mould culture filtrate gave, after 12–15 days incubation, a maximum inhibition of the growth of *Staph. aureus* at a dilution of 1:80 and, after this period, 37 flasks were harvested, yielding a total volume of culture filtrate of 11·4 l., pH 3·8–3·9. This filtrate, which was pale yellow in colour with a slight purple tint, was thoroughly extracted once, without further treatment, with an approximately equal volume of chloroform. The chloroform-extracted solution now showed no antibacterial activity. The chloroform solution was washed with a little water and was evaporated *in vacuo* to dryness giving 2·39 g. of a dark oil which was fractionated by the method of Menzel, Wintersteiner & Hoogerheide (1944). It was dissolved in ether (900 ml.), filtered from a trace of brown amorphous matter and extracted four times with 50 ml. of saturated aqueous $NaHCO_3$ solution each time (extract A). The ethereal solution was now re-extracted with a total of 100 ml. of 6% aqueous Na_2CO_3 solution in four portions (extract B).

The residual ether solution was washed with a little water, dried and evaporated to dryness giving a pale yellow oil which crystallized to a semi-solid mass (1·24 g.), and was crystallized from ethanol (5 ml.) giving finally 0·18 g. of colourless square-ended rods, m.p. 200° (decomp.). This substance, which contained nitrogen and sulphur, had the same properties and optical rotation as gliotoxin and did not depress the melting-point of an authentic specimen of this compound m.p. 200° (decomp.) kindly supplied by Dr J. R. Johnson of Cornell University, U.S.A.

The deep red coloured $NaHCO_3$ solution (extract A) was acidified and re-extracted with chloroform. This yielded, on evaporation, a small amount of a purple solid, the total weight of which was 83 mg. from 180 flasks. It was fractionally sublimed in a high vacuum and gave a crystalline purple sublimate, m.p. 201°, alone or admixed with authentic spinulosin. It also gave the same colour reactions with alkalis and concentrated sulphuric acid as are given by spinulosin. (Found: C, 52·7; H, 4·6. $C_8H_8O_5$ requires C, 52·2; H, 4·4%.)

The Na_2CO_3 solution (extract B) was acidified and extracted five times with an equal volume of benzene. Removal of the benzene *in vacuo* yielded only a trace of oil indicating the absence of helvolic acid.

DEHYDROCAROLIC ACID

Preparation

The culture, culture medium and cultural conditions used were the same as are described in the previous section, but it was found that the best yields of dehydrocarolic acid were obtained if malt agar slope cultures of the mould, incubated at 24° and not more than 8 days old, were employed. It is advisable to use a heavy sowing of mycelium and spores.

The optimum time of harvesting the flasks was 20–24 days (see Table 1, batches 6*a*–6*e*), by which time the glucose, as determined by polarimeter had fallen to less than 1·0%, the pH was 3·3–3·5 and the ferric chloride colour, obtained by adding 1 ml. of 10% aqueous $FeCl_3$ to 4 ml. of culture filtrate was a distinct orange brown. Under these conditions the yield of crude dehydrocarolic acid obtained averaged about 1·0 g./l. of culture filtrate.

Experimental details of a number of preparations are given in Table 1.

Table 1. *Production of dehydrocarolic acid*

Batch no.	No. of flasks	Incubation period (days)	Vol. of metabolism solution (l.)	pH	Residual glucose by polarimeter (%)	Total wt. of crude acid (g.)	Crude acid (g./l. of culture filtrate)
1*a*	20	12	6·25	3·8	1·89	7·71	0·68
1*b*	17	15	5·15	3·9	1·14		
2*a*	25	20	7·7	3·3	0·94	5·58	0·73
2*b*	25	21	7·65	3·3	0·63	7·01	0·92
2*c*	22	24	6·8	3·3	0·46	11·98	1·76
3*a*	25	20	7·6	3·3	0·73	8·8	1·16
3*b*	25	23	7·7	3·4	0·25	10·1	1·31
4	30	22	8·8	3·5	0·45	7·21	0·82
5	30	26	8·9	4·1	0·08	4·22	0·48
6*a*	15	12	4·75	3·7	1·97	1·50	0·32
6*b*	15	20	4·4	3·5	0·90	3·19	0·72
6*c*	30	24	8·6	3·7	0·37	6·35	0·75
6*d*	30	26	8·4	3·9	0·25	4·55	0·54
6*e*	27	34	7·2	4·8	Nil	2·69	0·37

The culture filtrate was acidified with conc. HCl (10 ml./l.) and extracted once with one-quarter of its volume of chloroform and then three times with one-eighth of its volume of chloroform each time. The combined chloroform extracts were evaporated *in vacuo* to 200–300 ml. This concentrate was shaken for a few moments with saturated $NaHCO_3$ solution (5 ml./l. of original culture filtrate) to remove spinulosin and other colouring matters. The chloroform solution, now almost colourless, was stirred vigorously with a mechanical stirrer for 1–1½ hr. with double the above quantity of saturated $NaHCO_3$ solution. At the end of this time all the dehydrocarolic acid had passed into the $NaHCO_3$ layer. It is a notable fact that dehydrocarolic acid is only slowly extracted from chloroform solution by aqueous $NaHCO_3$, the most probable explanation being that this substance is present in chloroform solution mainly in the form given in structure III and in $NaHCO_3$ solution as structure IV.

The $NaHCO_3$ solution was now acidified with conc. HCl and dehydrocarolic acid separated as almost colourless crystals which were washed with water and dried. The acid thus obtained is sufficiently pure for most purposes. Chloroform extraction of the filtrate and washings gave a second crop of somewhat less pure material. The sum of the weights of these two crops is referred to in Table 1 as 'crude' acid.

The further purification of dehydrocarolic acid is rendered more difficult by the ease with which the substance polymerizes particularly on heating in polar solvents. It can be recrystallized in small amounts from a large volume of light petroleum (b.p. 60–80°). For larger amounts the following method proved to be satisfactory. The finely powdered dry acid (12 g.) was added to a boiling mixture (1·5 l.) of chloroform (17–20% by vol.) and light petroleum (b.p. 60–80°), Δ of mixed solvents, 0·822–0·851 at 20°. After boiling for a few moments under reflux with vigorous agitation the solution was quickly filtered and cooled. 2·5 g. of the crude acid remained undissolved and there separated from the filtrate 7·5 g. of pure dehydrocarolic acid as colourless fine platelets. Evaporation *in vacuo* of the mother liquors to one-third vol. yielded an only slightly impure second crop.

Isolation of carlosic acid

The culture filtrate from batches 1*a* and 1*b*, Table 1 (11·4 l.) after separation of dehydrocarolic acid with chloroform, was re-extracted five times with ether, using 2 l. of solvent each time. On removal of the solvent there remained 0·76 g. of a reddish semi-crystalline residue. This was dissolved

in dry ether and fractionally crystallized by the graded addition of light petroleum (b.p. 60–80°). An almost colourless crystalline fraction (0·08 g.; m.p. 178°) was obtained and was recrystallized from benzene from which it separated in colourless needles identical in form with those of authentic carlosic acid crystallized in the same way. This substance was identified as carlosic acid as follows: m.p. 178°, not depressed on admixture with authentic carlosic acid. Rotation of free acid ($c = 0{\cdot}99\,\%$) in water: $[\alpha]_{5461}^{19°} = -160°$, unchanged overnight; carlosic acid has the same rotation, $-160°$ (Clutterbuck *et al.* 1934). (Found: C, 52·51; H, 5·26; equivalent by titration, 113·6. $C_{10}H_{12}O_6$ requires C, 52·61; H, 5·31 %; equivalent as a dibasic acid, 114.)

Properties of dehydrocarolic acid

Dehydrocarolic acid crystallizes in colourless fine platelets. It has no melting-point, since because of its great tendency to polymerize, it changes to a glassy or rubbery material on heating at temperatures above 80° whether alone or in many solvents, particularly polar solvents. Its empirical formula is $C_9H_8O_4$. (Found: C, 59·95, 59·95; H, 4·58, 4·41; neutralization equivalent to phenolphthalein (sharp end-point), 181·1. $C_9H_8O_4$ requires C, 59·98; H, 4·47 %; equivalent, titrating as a monobasic acid, 180·2.) An aqueous solution of the sodium salt ($c = 0{\cdot}50\,\%$) is optically inactive. The substance does not inhibit the growth of *Staph. aureus* (L.S.H.T.M. strain) at a concentration of 1:500.

It is almost insoluble in light petroleum, slightly soluble in ether, ethanol and ethyl acetate but dissolves readily in cold chloroform though it is not quite so soluble in this solvent as is carolic acid.

It dissolves to the extent of about 2 % in cold water and this aqueous solution gives the following reactions. (*a*) It is strongly acidic turning Congo red paper blue. (*b*) With $FeCl_3$ a characteristic bright orange colour. (*c*) No colour with $NaNO_2$ solution. (*d*) It readily reduces Fehling's solution, immediately decolorizes $KMnO_4$ solution and fairly quickly gives a precipitate with Brady's reagent, 0·3 % 2:4-dinitrophenylhydrazine in 2N-HCl. A neutral 0·1N-aqueous solution of the sodium salt gives the following reactions. (*a*) With $CaCl_2$ or $BaCl_2$, no precipitate even after the addition of 4 vol. of ethanol. (*b*) $AgNO_3$ or $HgCl_2$, no precipitate but some darkening overnight. (*c*) Lead acetate, slight precipitate on standing. (*d*) Basic lead acetate, heavy white precipitate. (*e*) Copper sulphate, nickel sulphate or uranyl acetate, no precipitate but on standing overnight well formed nodular crystals develop, blue, blue-green and yellow respectively.

On warming with conc. H_2SO_4, dehydrocarolic acid dissolves giving a colourless solution which, on heating to 120°, becomes a deep bluish-red colour which is almost identical with that given by carolic acid at 140°. Both solutions show an intense brownish red fluorescence in ultra-violet light.

Dehydrocarolic acid mono-2:4-dinitrophenylhydrazone

Brady's reagent (0·3 % 2:4-dinitrophenylhydrazine in 2N-HCl; 160 ml.) was added to a solution of dehydrocarolic acid (0·5 g.) in water (50 ml.) acidified with conc. HCl (5 ml.). A yellow crystalline precipitate separated and was collected after 24 hr. (0·50 g.; m.p. 152°). Recrystallized from ethanol (25 ml.) it was obtained as clusters of yellow platelets (0·32 g.; m.p. 157° unchanged on further crystallization). The substance reacts acid to litmus and is at once soluble in very dilute Na_2CO_3 solution. An ethanolic solution gives with a trace of alkali a red colour characteristic of a mono-2:4-dinitrophenylhydrazone (Neuberg's reaction). Carolic acid, when treated with Brady's reagent under exactly the same conditions, gives no precipitate for some hours but finally yields a small amount of a dirty brown deposit. (Found: C, 47·4; H, 4·0; N, 14·6; equivalent by titration (Clift & Cook, 1932), 380. $C_{15}H_{14}O_8N_4$ requires C, 47·6; H, 3·7; N, 14·8 %; equivalent as a monobasic acid 378. $C_{15}H_{12}O_7N_4$ requires C, 50·0; H, 3·3; N, 15·6 %; equivalent, 360.) Hence this derivative is the 2:4-dinitrophenylhydrazone of the hydrated form of dehydrocarolic acid, structure IV on p. 569.

Catalytic reduction of dehydrocarolic acid. Formation of dl*-carolic acid*

Dehydrocarolic acid (1 g.) dissolved in water (80 ml.) was reduced with hydrogen by means of a catalyst prepared from $PdCl_2$ (0·2 g.) and norite charcoal (0·5 g.), the method used being that described by Clutterbuck *et al.* (1935*a*). 125 ml. of hydrogen was absorbed in 4 min. (theory for uptake of $1H_2 = 124{\cdot}4$ ml.) when absorption of hydrogen had become very slow. The catalyst was now removed by filtration and the filtrate was extracted three times with one-third its volume of chloroform. On removal of the solvent *in vacuo* there remained 0·85 g. of white solid, m.p. 95–105°. On continuous ether extraction of the chloroform-extracted solution, 0·10 g. of an oil was obtained which gave a red $FeCl_3$ reaction in water similar to that recorded for tetrahydrocarolic acid by Clutterbuck *et al.* (1935*a*). Larger quantities of the reduction product were more conveniently prepared as follows: dehydrocarolic acid (5 g.) was suspended in water and brought into solution by the addition of 0·2N-NaOH (112 ml.); total vol. 150 ml. This was reduced in 10 min. by a catalyst prepared from $PdCl_2$ (0·5 g.) and norite (1 g.). Uptake of hydrogen, 78 % of theory; 3·70 g. (74 % of theory) of reduction product

was isolated after acidification and extraction with chloroform. Catalytic reduction of the fully neutralized sodium salt of dehydrocarolic acid is slow but gives about the same yield of reduction product.

The reduction product (10 g.) was purified by dissolving in warm benzene (40 ml.) and adding warm dry ether (100 ml.). On repeated recrystallization colourless, globular, hard clusters of needles or plates were obtained. (Found: C, 59·57; H, 5·60. $C_9H_{10}O_4$ requires C, 59·31; H, 5·54 %.) The reduction product showed a similar anomalous behaviour in respect to melting-point as was recorded for *d*-carolic acid from *Penicillium Charlesii* by Clutterbuck *et al.* (1935*a*) with which it was compared. Reduction product, m.p. 117°; *d*-carolic acid, m.p. 130°; mixture of the two, m.p. 121°. All three samples reset on cooling and all remelted at 113°.

The reduction product is optically inactive. It gives chemical reactions, e.g. $FeCl_3$ colour, conc. H_2SO_4 colour, reactions with metallic salts, which are identical with those given by *d*-carolic acid when tested side by side. It is however more soluble in all solvents.

Its identity as dl-*carolic acid* was established by a study of its behaviour on acid hydrolysis since it gives the same hydrolytic products (CO_2, acetoin, and γ-butyrolactone) and in the same amounts (approximately one molecule of each) as does *d*-carolic acid. The reduction product (1·008 g.) was hydrolyzed by boiling its solution in 2N-H_2SO_4 (50·0 ml.) under nitrogen for 3 hr. Unlike dehydrocarolic acid (next section) there was little formation of colour and no evidence of polymerization. The method of estimating the hydrolytic products was that described in detail by Clutterbuck *et al.* (1935*a*, p. 313) and the following results were obtained.

(*a*) *Carbon dioxide*: equivalent to 10·28 ml. N-HCl, or 92·6 % of 1 mol.; *d*-carolic acid gave 95·4 %.

(*b*) *Acetoin*: confirmed qualitatively by strong reduction of Fehling's solution in the cold, a strong Voges-Proskauer reaction and the slow formation of the bis-dinitrophenylhydrazone of diacetyl on treatment with 2:4-dinitrophenylhydrazine in 2N-HCl; m.p. and mixed m.p. 318°. Estimation by the Wood-Ost method gave 98 % of 1 mol.; *d*-carolic acid gave 82·6 %.

(*c*) *γ-Butyrolactone*: confirmed qualitatively by micro-distillation of the lactone (0·50 g.) (see next section) and conversion into the phenylhydrazide, m.p. 94°, alone or admixed with authentic γ-hydroxy-*n*-butyric acid phenylhydrazide. It was estimated quantitatively by hot titration of the hydrolysis solution which had been previously neutralized in the cold: found, 105·4; *d*-carolic acid gave 100·7 %.

Thus acid hydrolysis of the reduction product proceeded exactly as for *d*-carolic acid according to the equation:

$$C_9H_{10}O_4 + 2H_2O \rightarrow \underset{\text{acetoin}}{CH_3.CHOH.CO.CH_3} + \underset{\text{butyrolactone}}{CH_2.CH_2.CH_2.CO} + CO_2$$

Acid hydrolysis of dehydrocarolic acid

Dehydrocarolic acid (2·036 g.) was hydrolyzed by boiling for 3 hr. with 2N-H_2SO_4 (100 ml.) in a current of CO_2-free nitrogen. The issuing gas was passed directly into a flask cooled in a freezing mixture, then through a bubbler containing Brady's reagent and finally through a bubbler containing standard baryta.

The dehydrocarolic acid dissolved when the bath temperature reached 70° giving initially a clear solution which, on raising the temperature, rapidly became brown and from which there quickly separated a dark brown, rubbery, polymerized product (total, 0·4 g.). Yellow vapours of diacetyl appeared in the upper portion of the hydrolysis flask and a yellow liquid, later setting to a yellow crystalline mass collected in the flask in the freezing mixture. Further, a heavy yellow precipitate quickly appeared in the bubbler containing Brady's reagent. Under similar conditions of hydrolysis neither *d*- nor *dl*-carolic acid gives any polymerized product, nor any appreciable colour, nor any precipitate in the Brady bubbler.

Identification and estimation of the products of hydrolysis gave the following results:

(*a*) *Carbon dioxide*: equivalent to 15·80 ml. N-HCl, or 92 % of 1 mol. if allowance is made for the weight of insoluble polymerized product formed.

(*b*) *Diacetyl*. The contents of the flask in the freezing mixture were diluted with a little water and there was added hydroxylamine hydrochloride (1·6 g.) and sodium acetate crystals (3·2 g.). A crystalline solid (0·28 g.) quickly separated which, after recrystallization from ethanol (7 ml.) gave colourless, thick, broad rods, wt. 0·17 g., m.p. 246° alone or on admixture with authentic dimethylglyoxime. (Found: C, 41·22; H, 6·97; N, 23·8. $C_4H_8O_2N_2$ requires C, 41·37; H, 6·94; N, 24·1 %.) A very dilute aqueous solution gave, on addition of nickel sulphate, the characteristic brilliant scarlet precipitate of nickel dimethylglyoxime. The heavy yellow precipitate in the Brady bubbler was collected after 3 days; wt. 0·44 g.; m.p. 318° decomp., alone or on admixture with authentic diacetyl bis-2:4-

dinitrophenylhydrazone. It gave a deep blue colour with Neuberg's reagent which is typical of a bis-2:4-dinitrophenylhydrazone.

The weights obtained of dimethylglyoxime and of diacetyl bis-2:4-dinitrophenylhydrazone correspond to 0·32 g. of diacetyl or 41 % of 1 mol. if allowance is made for the weight of insoluble polymerized product formed.

(c) *γ-Butyrolactone.* The cooled, brown, acid hydrolysis solution was filtered from the brown polymerized product and the filtrate was exactly neutralized in the cold with 2N-NaOH to phenolphthalein. It was then heated and neutralization continued to a permanent end-point, requiring 5·30 ml. N-NaOH or 60 % of 1 mol. of γ-butyrolactone if allowance is made for the weight of insoluble polymerized product formed.

The neutralized solution was now evaporated *in vacuo* to dryness several times. Only a faint trace of volatile product was detectable in the distillate either by the Voges-Proskauer test or with Brady's reagent indicating the absence of acetoin; cf. *d-* or *dl*-carolic acid.

The solid remaining after evaporation was dissolved in water (50 ml.), made strongly acid with 5N-H_2SO_4 and boiled for a few minutes. The small amount of polymerized product which was formed was separated by filtration and the clear yellow filtrate was continuously extracted with ether for 2 days. On removal of the ether there remained 0·79 g. of a clear yellow oil which was distilled at atmospheric pressure in a micro-distillation apparatus. The distillate, a clear yellow mobile oil, wt. 0·53 g., b.p. 195–200° (γ-butyrolactone boils at 203°) was treated with phenylhydrazine (0·66 g.) in ethanol (10 ml.) and the mixture was boiled under reflux for 2 hr. The ethanol was removed *in vacuo* and the semi-solid residue was crystallized from ethyl acetate giving colourless, lustrous plates, m.p. (after sublimation in a high vacuum) 96° alone or admixed with the phenylhydrazide prepared from authentic γ-butyrolactone. (Found: C, 61·84; H, 7·61; N, 14·1. $C_{10}H_{14}O_2N_2$ requires C, 61·83; H, 7·27; N, 14·4 %.)

Thus acid hydrolysis of dehydrocarolic acid proceeds, but not quantitatively, according to the equation:

$$C_9H_8O_4 + 2H_2O \rightarrow CH_3.CO.CO.CH_3 + CH_2.CH_2.CH_2.CO + CO_2$$

diacetyl — butyrolactone

and shows that the extra centre of unsaturation in dehydrocarolic acid, as compared with carolic acid, cannot be in the butyrolactone side chain.

Action of ozone on dehydrocarolic acid. Formation of formaldehyde

Dehydrocarolic acid (0·205 g.) was dissolved in water (25 ml.) cooled in ice and a stream of ozonized oxygen was passed through for 3 hr. No formaldehyde could be detected in the issuing gases. The ozonized solution was left at room temperature overnight in a closed flask. The formaldehyde was precipitated direct from the solution by the method of Reeves (1941). After the addition of M-sodium acetate solution (22 ml.) and alcoholic dimedone solution (11 ml. of 8 % solution) the dimedone compound of formaldehyde separated at once. It was collected, washed with water and dried *in vacuo*; wt., 0·210 g. or 64 % of theory; m.p. 187–188°. It was recrystallized from ethanol (10 ml.) giving colourless, shining broad needles, m.p. 189°, alone or in admixture with the authentic formaldehyde dimedone compound, methylene-bis-dimethyldihydroresorcinol. (Found: C, 69·80; H, 8·30. $C_{17}H_{24}O_4$ requires C, 69·85; H, 8·25 %.)

It is thus clear that dehydrocarolic acid contains the $CH_2{=}C$ grouping.

SUMMARY

1. *Dehydrocarolic acid*, $C_9H_8O_4$, a hitherto undescribed mould metabolic product, is formed on Czapek-Dox glucose medium by a strain of *Penicillium cinerascens* Biourge. Its isolation, properties and breakdown products are described. It has been shown to be a dehydro derivative of carolic acid, $C_9H_{10}O_4$, a metabolic product of *P. Charlesii* G. Smith. Dehydrocarolic acid hydrate has been shown to be α-(γ-hydroxy-*n*-butyryl)-γ-methylenetetronic acid.

2. Culture filtrates of *P. cinerascens* show antibacterial activity due to the presence of the antibiotics gliotoxin and spinulosin. Dehydrocarolic acid has not any appreciable antibacterial activity.

3. Carlosic acid, previously reported as a metabolic product of *P. Charlesii* G. Smith, was also isolated in small amounts.

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The Action of Vitamin K in Hypervitaminosis A

By S. E. WALKER, E. EYLENBURG and T. MOORE
Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

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Moore & Wang (1943, 1945) have given a comprehensive review of the toxic effects produced in experimental animals by greatly excessive amounts of vitamin A. From their experiments with pure vitamin A acetate they concluded that the most characteristic lesions were skeletal fractures and haemorrhage, the first of these lesions being predominant in growing animals and the second in adult animals. The fractures caused by pure vitamin A have been confirmed by Herbst, Pavcek & Elvehjem (1944) and Pavcek, Herbst & Elvehjem (1945) who also found that telangiectatic bovine livers, rich in vitamin A, are toxic to rats. Light, Alscher & Frey (1944) approached the problem from a new angle, by showing that hypervitaminosis A in rats was associated with a pronounced hypoprothrombinaemia, which could be corrected by giving vitamin K as 2-methyl-3-phytyl-1:4-napthaquinone. The object of the present investigation has been to confirm and extend this important observation.

EXPERIMENTAL

Method

In a series of experiments, piebald rats were fed on massive doses of vitamin A for periods varying from 10 to 18 days, with or without the addition of vitamin K, or, in one experiment, of vitamin C. The source of the vitamin A was halibut-liver oil, containing 40,000 i.u./g., which was kindly supplied by Allen and Hanbury, Ltd. At the termination of each experiment the rats were killed under ether anaesthesia. The chest wall was opened and as much blood as possible withdrawn from the heart into an oxalated syringe. The livers were removed, weighed, and placed in 5% KOH solution, and the bodies were X-rayed for the presence of fractures. Vitamin A was estimated in the livers by the antimony trichloride method applied after the digestion of the tissues by alkali (Davies, 1933) and in the blood by the same method applied according to the technique of Yudkin (1941). Plasma clotting times were measured by the method of Campbell, Smith, Roberts & Link (1941) and an approximate measurement was made of the plasma/cell ratio by centrifuging the blood for a standard time in graduated centrifuge tubes. Unfortunately no haematocrit was available.

The action of vitamin K in hypervitaminosis A

Exp. 1–4. Piebald rats of both sexes, usually young but in one experiment nearing maturity, were given a basal diet of casein 20%, sugar 60%, fat 15% and salt mixture 5%, with dried yeast 10% (additional). Vitamin A was supplied as one drop of halibut-liver oil/rat/week, vitamin D as one drop of 'Radiostol', and vitamin E as 1 mg. of *dl*-α-tocopheryl acetate. Each experiment, except no. 4, included three groups which are described in Tables 1 and 2 as 'control', 'excess of vitamin A' and 'excess of vitamin A + vitamin K'. In the control groups the fatty component of the basal diet was supplied as arachis oil, whereas groups given excess of vitamin A received their fat as halibut-liver oil, which was freshly mixed each day with the solid components of the diet. In Exp. 1 the group given vitamin K in addition to vitamin A received it in the form of 'Synkavit' (tetrasodium 2-methyl-1:4-naphthahydroquinone diphosphoric acid ester). The Synkavit was added to the dry constituents of the diet so as to supply about 160 μg./rat daily. To avoid any possible criticism that the admixture

with the diet was uneven, however, vitamin K was given in subsequent experiments as 80 μg. of 2-methyl-1:4-naphthaquinone three times weekly, the dose being administered in oily solution to each animal by dropping pipette.

Since the lesions caused by excess of vitamin A vary with the age of the animal the sizes of the animals used in the various experiments are relevant. The rats used in Exps. 1 and 2 had initial body weights of 43–85 g., and may be described as 'young' rats. The rats used in Exp. 3 had weights of 125–153 g., and may be described as 'adolescent'. The rats used in Exp. 4 were 'young' rats of weights 83–126 g. Exps. 2 and 3 were carried out simultaneously, and the results for the young and adolescent rats in these experiments are therefore strictly comparable. In Exps. 1–3 the animals were killed without previous fasting, but in Exp. 4 (Table 3) the animals were fasted for 24 hr. before being killed.

RESULTS

As already stated the most characteristic lesions in hypervitaminosis A appear to be haemorrhages, variable in intensity and distribution, and also spontaneous skeletal fractures, which occur mainly in young rats. Both these injuries were produced in various groups in the present experiments, and the apparent effect of age was confirmed. Thus at autopsy the haemorrhages found in adolescent animals of Exp. 3 were more widespread and severe than those in young rats, but spontaneous skeletal fractures were absent. The only evidence of softness in the bones in the adult groups was that in two instances fractures were produced accidentally while

Table 1. *Effect of vitamin K on hypervitaminosis A in young rats*

		Rat			Vitamin A: Liver		Vitamin A: Blood	Plasma prothrombin			
Exp.		No.	Wt.* (g.)	Days on exp.	Concentration (i.u./g.)	Total (i.u.)	concentration (i.u./100 ml.)	time (sec.)	Plasma/Cell ratio	Haemorrhages†	Fractures†
1.	Controls	1	72	12	144	760	74	31	1·2	0	0
		2	65	18	145	530	67	31	1·2	0	0
				Means	**144**	**650**	**71**	**31**	**1·2**		
1.	Excess of vit. A	3	80	18	9,200	42,000	240	100	2·1	+	0
		4	66	12	11,100	49,000	890	29	3·0	0	+ + +
		5	70	18	4,400	18,000	250	35	2·6	+	+ +
		6	82	18	5,300	24,000	290	143	2·7	0	+ +
		7	66	18	13,500	49,000	240	45	2·0	+	+ + +
				Means	**8,700**	**36,000**	**364**	**70**	**2·5**		
1.	Excess of vit. A + vit. K	8	75	18	3,600	10,800	2,050	32	2·2	+ +	+
		9	64	12	8,100	26,000	4,960	29	2·0	+	+ + +
		10	79	18	7,100	27,000	130	32	1·8	0	+ + + +
		11	79	18	4,500	18,000	210	30	2·3	0	+
		12	81	12	7,100	25,000	1,330	29	2·0	0	+ +
				Means	**6,100**	**21,000**	**1,740**	**30**	**2·1**		
2.	Controls	13	51	17	300	1,410	84	38	1·5	0	0
		14	43	17	140	680	90	41	0·9	0	0
		15	50	10	94	260	30	C	—	0	0
		16	49	10	186	520	50	28	1·4	0	0
				Means	**180**	**720**	**64**	**36**	**1·3**		
2.	Excess of vit. A	17	85	17	1,500	8,400	400	101	3	+	+ + +
		18	70	10	4,800	21,000	470	81	2	+	+ +
		19	70	10	2,600	15,200	1,330	68	2	0	+
		20	70	17	9,500	46,000	350	354	2·2	0	+ +
		21	69	10	8,800	37,000	140	60	1·6	0	+ +
		22	78	17	12,700	62,000	640	51	2·2	0	+ + +
				Means	**6,700**	**32,000**	**555**	**119**	**2·2**		
2.	Excess of vit. A + vit. K	23	51	12D	4,600	13,900	—	—	—	0	+ + +
		24	76	17	8,800	35,000	1,550	32	1·5	+	+ + + +
		25	77	10	6,000	26,000	300	25	1·3	0	+ + +
		26	66	10	7,100	33,000	1,760	24	1·6	0	+ + +
		27	64	17	4,700	21,000	1,010	31	2·3	+	+ + +
		28	64	10	4,300	17,500	440	27	1·7	0	+ +
				Means	**5,900**	**24,400**	**1,010**	**28**	**1·7**		

* Initial weight.

† The number of crosses indicates the general intensity of haemorrhage or the number of fractures.

C = clotted on withdrawal. D = died.

Table 2. *Effect of vitamin K on hypervitaminosis A in adolescent rats*

		Rat			Vitamin A: Liver		Vitamin A: Blood	Plasma prothrombin			
Exp.	No.	Wt.* (g.)	Days on exp.	Concentration (i.u./g.)	Total (i.u.)	concentration (i.u./100 ml.)	time (sec.)	Plasma/Cell ratio	Haemorrhages†	Fractures†
3. Controls	29	193	10	25	205	69	27	0·9	0	0
	30	165	10	28	196	50	32	0·8	0	0
	31	200	17	56	515	100	44	0·8	0	0
	32	196	17	98	1,010	100	34	1·3	0	0
			Means	**52**	**482**	**80**	**34**	**1·0**		
3. Excess of vit. A	33	190	9	5,600	50,000	290	202	2·8	+ + +	0
	34	173	10	5,900	48,000	80	226	3	+	0
	35	168	17D	7,200	88,000	—	—	—	+ + +	+ +‡
	36	190	10	5,200	41,000	60	89	2·2	+ + +	0
	37	151	17	12,300	96,000	380	101	1·1	0	0
	38	144	17	11,400	92,000	220	281	1·3	+	0
			Means	**7,900**	**69,000**	**206**	**180**	**2·1**		
3. Excess of vit. A + vit. K	39	127	10	8,200	56,000	210	27	1·3	0	0
	40	140	17	11,800	87,000	310	33	1·2	0	0
	41	153	17	14,100	97,000	700	30	1·4	0	0
	42	133	10	8,000	55,000	150	27	1·2	0	0
	43	143	10	7,600	56,000	130	28	0·9	0	0
	44	125	17	11,300	74,000	270	30	1·6	0	+‡
			Means	**10,200**	**71,000**	**295**	**29**	**1·3**		

* Initial weight.
† The number of crosses indicates the general intensity of haemorrhage or the number of fractures.
‡ Sustained during handling post-mortem. D = died.

Table 3. *Effect of vitamin K and of vitamin C on hypervitaminosis A in young rats fasted for 24 hr. before being killed*

		Rat			Vitamin A: Liver		Vitamin A: Blood	Plasma prothrombin			
Exp.	No.	Wt.* (g.)	Days on exp.	Concentration (i.u./g.)	Total (i.u.)	concentration (i.u./100 ml.)	time (sec.)	Plasma/Cell ratio	Haemorrhages†	Fractures†
4. Excess of vit. A	45	131	18	22,000	54,000	145	68	—	+	0
	46	93	18	27,000	117,000	126	37	—	0	+ +
	47	95	18	15,800	48,000	133	76	—	+	+ +
	48	110	18	23,000	70,000	370	52	—	+	+
	49	98	18	18,800	66,000	242	—	—	+	+ +
	50	103	18	8,400	42,000	144	90	—	0	+ +
			Means	**19,200**	**66,000**	**193**	**65**			
4. Excess of vit. A + vit. K	51	91	18	9,400	42,000	96	35	—	0	+ +
	52	86	18	12,700	48,000	152	37	—	0	+ +
	53	126	18	14,500	86,000	104	32	—	0	+ +
	54	92	18	12,800	50,000	363	—	—	0	+ +
	55	83	18	27,000	68,000	160	—	—	0	+ +
	56	89	18	9,300	28,000	211	38	—	0	+ + +
	57	88	18	10,700	39,000	144	30	—	0	+ +
			Means	**13,800**	**52,000**	**176**	**34**			
5. Excess of vit. A + vit. C	58	88	18	23,200	70,000	142	—	—	+	+ +
	59	106	18	9,900	44,000	122	46	—	+	+ +
	60	92	18	10,200	41,000	167	42	—	0	+ +
	61	95	18	12,600	44,000	542	68	—	+	0
	62	94	18	14,900	62,000	120	—	—	0	+ +
	63	85	18	10,300	36,000	139	45	—	0	+ +
	64	86	18	14,400	53,000	291	47	—	+	+ +
			Means	**13,600**	**50,000**	**218**	**50**			

* Initial weight.
† The number of crosses indicates the general intensity of haemorrhage or the number of fractures.

the animals were being handled after death. During the course of the experiments the older animals seemed to be in a poorer state of health than the young ones, and their food consumption, and hence their intake of vitamin A, was smaller. This may account in part for the difference in the reactions of the younger and older rats to excess of vitamin A.

The recent claim that hypervitaminosis A is accompanied by hypoprothrombinaemia, as indicated by a prolonged plasma clotting time, was confirmed in all groups given halibut-liver oil without vitamin K. The administration of vitamin K either as 2-methyl-1:4-naphthaquinone or as 'Synkavit' prevented this abnormality. The effect of vitamin K on haemorrhage was somewhat less clear cut. In adolescent rats which were given vitamin K (Exp. 3) no haemorrhages were found, but the results were less definite in the younger animals. On the other hand vitamin K had no effect on the skeletal lesions. Spontaneous fractures occurred in all young rats given excess of halibut liver oil, and accidental fractures after death in one animal in each of the groups of adult rats, irrespective of whether vitamin K was given or not.

Our original purpose in estimating vitamin A in the livers and tissues was to obtain information as to the levels which were associated with the development of toxic symptoms. The results for Exps. 1–3, in which the rats were killed without preparatory fasting, indicate the prevailing levels at the conclusion of the experiment, as influenced by the usual habits of the animals in ingesting their food. The values for liver in rats receiving excess of vitamin A were of the same order as those found in hypervitaminotic rats by Davies & Moore (1934). The values for blood plasma were much higher than in control rats. The decision to fast the animals in Exp. 4 before killing was taken since it appeared from the results of Exps. 1 and 2 that vitamin K might possibly have the effect of raising the level of vitamin A in the plasma and reducing the amount stored in the liver. In attempting to confirm this effect it seemed desirable to eliminate the possibility of large temporary variations in the plasma vitamin A, which might be expected from experience with other animals to occur within the first few hours after ingesting a massive dose. After this precaution the mean vitamin A contents of the plasma of animals treated or not treated with vitamin K were found to be virtually identical, while the difference between the mean liver reserves was small in relation to the wide variations which occurred within each group.

Another effect which has not been reported previously was found in rats given excess of vitamin A. On withdrawal their blood appeared to be thin and watery. The mean plasma/cell ratio for all animals given excess of vitamin A without vitamin K was 2·2, as compared with 1·2 for control animals. When vitamin K was given in addition to vitamin A the mean ratio was 1·6.

The action of vitamin C in hypervitaminosis A

Vedder & Rosenberg (1938) claimed that ascorbic acid is beneficial to rats given excess of jew-fish-liver oil, a rich source of vitamin A. Moore & Wang (1945), however, failed to improve the condition of rats receiving excess of vitamin A acetate by giving them large doses of ascorbic acid. In the present investigation it seemed of interest to re-examine this point, with particular reference to the effect of ascorbic acid on the prothrombin time.

Exp. 5. This experiment was carried out simultaneously and in parallel with Exp. 4. A group of rats, receiving excess of halibut-liver oil, was dosed with 50 mg. of ascorbic acid/rat daily by dropping pipette. Table 3 indicates that the ascorbic acid gave no protection against either haemorrhages or fractures. The mean prothrombin time was less than in the untreated group, but greater than in the group dosed with vitamin K.

Dicumarol poisoning

Further evidence that skeletal fractures and hypoprothrombinaemia may be produced separately was obtained in experiments with dicumarol. This substance is known to produce a marked fall in blood prothrombin levels in various species (Overman, Stahmann, Sullivan, Huebner, Campbell & Link, 1942). Therefore it was considered desirable to investigate whether the hypoprothrombinaemia of dicumarol poisoning was accompanied by any bony changes which might produce fractures.

Exp. 6. Twelve young rats of weights ranging from 36 to 55 g. were given daily doses of dicumarol varying from 1 to 3 mg. Their diet was identical with that of the controls in the experiments described above, the dicumarol being added to the food. The animals died within 3–7 days and at autopsy severe and extensive haemorrhages were found. Careful examination was made of the limbs but no fractures or visible bone abnormalities were seen. As the survival time was so short the experiment was repeated, with smaller doses calculated on a body-weight basis and slightly older rats (80–97 g.) in order to observe the effect of a more prolonged action of dicumarol. The doses in this instance were from 1·5 to 5 mg., and the survival times 10–38 days. The post-mortem findings were similar to those in the preceding experiment, and again no fractures were found.

DISCUSSION

In confirmation of the results of Light, Alscher & Frey (1944) we have found that an injurious excess of vitamin A causes prolongation of the plasma

clotting time in rats, which is presumed to indicate a condition of hypoprothrombinaemia. This abnormality could be corrected by the administration of vitamin K. It would appear therefore that toxic excess of vitamin A induces a secondary deficiency of vitamin K. There is a variety of ways in which this could arise. Since, on ordinary experimental diets, the rat can exist without any extraneous source of vitamin K it might be considered that the excess of vitamin A interferes with the synthesis of vitamin K by the intestinal flora. Another possibility is that vitamin A in toxic doses may interfere with the absorption either of small amounts of vitamin K which may be present in some unsuspected source in the basal diet, or of the vitamin K which has been synthesized in the gut. Yet another mechanism might be the destruction or inactivation of the vitamin K within the liver or elsewhere in the body. Finally, it is conceivable that excessive amounts of vitamin A or its metabolites might in some way increase the rat's demand for vitamin K.

Although rats given vitamin K invariably had normal prothrombin times, the action of vitamin K in preventing haemorrhage was consistent only in rats which had initial body weights of about 85 g. and over. Thus out of 6 rats in Exp. 3 and 7 rats in Exp. 4 which were dosed with vitamin K, none had haemorrhage. Among the smaller rats used in Exps. 1 and 2, however, 4 which were dosed with vitamin K had haemorrhage. Moreover, two of those not dosed with vitamin K which had haemorrhage gave normal prothrombin times.

In agreement with previous work (Moore & Wang, 1945) excess of vitamin A also caused spontaneous fractures of the bones in young, but not in adolescent, rats. It may be noted that the division according to body weight which determined the incidence of fractures was not the same as that which was found for the action of vitamin K in preventing haemorrhage. Thus the rats in Exp. 4 which sustained fractures were nevertheless protected against haemorrhage by vitamin K. In no group of young rats given halibut-liver oil was the incidence of fractures reduced by dosing with vitamin K. It appears, therefore, that the production of fractures in hypervitaminosis A bears no close relationship to the delayed clotting times. The results of the dicumarol experiments give further evidence that hypoprothrombinaemia can exist as a separate entity without the bones being visibly involved. The hypoprothrombinaemia of dicumarol poisoning can also be cured by vitamin K (Overman, Field, Baumann & Link, 1942).

It might be thought that the increase in the plasma/cell ratios found in the hypervitaminotic rats was only due to blood dilution subsequent to bleeding and not to a specific action of excess vitamin A. In some young rats, however, an increase in the plasma/cell ratio was not accompanied by haemorrhage. The observations that hypervitaminosis A in rats is accompanied by an increased plasma/cell ratio is in agreement with the work of Poumeau-Delille (1943) who found a severe erythroblastic anaemia in rats given toxic doses of vitamin A. In these animals there was a marked fall in both red and white blood cells, although the platelet count was normal. Poumeau-Delille states that there was no alteration in bleeding or coagulation times. This statement does not agree with the conclusions of the present work, but it must be noted that the animals in the French laboratory were given halibut-liver oil by subcutaneous injection, whereas oral administration was employed in the present investigation.

There is little in the literature suggesting any relationship between vitamins K and A in the normal animal. Clayton & Baumann (1944), studying the effect of various substances on the rate of depletion of hepatic vitamin A in rats, found that vitamin K had a slight retarding influence. As the anti-coagulant dicumarol was shown to have a similar effect on vitamin A reserves this action can hardly be regarded as specific for vitamin K. Blood and liver vitamin A levels in young chicks suffering from vitamin K deficiency were measured by Tomaszewski & Engel (1939) but their results were inconclusive.

SUMMARY

1. In addition to the well known lesions of hypervitaminosis A in rats, namely skeletal fracture and haemorrhage, the presence of a marked hypoprothrombinaemia has been confirmed.

2. The administration of vitamin K prevented this hypoprothrombinaemia and diminished the incidence of haemorrhage, although the incidence of fractures remained unchanged.

3. Massive doses of ascorbic acid did not prevent the fractures or haemorrhage in hypervitaminosis A.

4. Hypoprothrombinaemia induced in rats by dicumarol poisoning, in the absence of any excess vitamin A, was not associated with visible skeletal changes.

5. Massive doses of vitamin A caused an increase in plasma/cell ratios, which could sometimes be controlled by vitamin K.

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Flocculation Tests with Electrophoretically Separated Serum Proteins

BY N. F. MACLAGAN AND D. BUNN,* *The Department of Chemical Pathology, Westminster Hospital Medical School, London, S.W. 1, and the National Institute for Medical Research, Hampstead, London, N.W. 3*

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There are now a number of flocculation tests carried out on blood serum which have proved useful indicators of hepatic dysfunction, and the mechanism involved in these tests is becoming much clearer as a result of American work with electrophoretically separated proteins. Thus the γ-globulin is very active in precipitating the gold and cephalin-cholesterol reagents (Gray, 1942; Kabat, Moore & Landow, 1942) and this fraction is also known to be increased in the serum from patients with various types of hepatitis (Gray & Barron, 1943). In both these tests albumin has an inhibitory effect and, moreover, albumin from hepatitis serum has less inhibitory power than albumin from normal serum (Kabat, Hanger, Moore & Landow, 1943; Moore, Pierson, Hanger & Moore, 1945). Very little information is available as to the effect of the α- and β-globulin fractions.

The thymol turbidity test (Maclagan, 1944*a*) appeared to be closely related to the above two tests both on clinical and chemical grounds, and the precipitate formed in the reaction was shown to be a globulin-thymol-phospholipid complex. It therefore seemed probable that a similar mechanism was operating in this test. The experiments reported here were undertaken to elucidate this point, and to investigate the α and β fractions more fully. In the meantime, Recant, Chargaff & Hanger (1945) and McCord (1945) have failed to produce a turbidity when the thymol reagent was added to pure γ-globulin solutions. A preliminary report of our experiments has already appeared (Maclagan & Bunn, 1946).

* Present address, Department of Chemistry, The University, Leeds.

MATERIAL

The results reported here were obtained with two collections of human serum: *N*, 40 ml. from a normal subject; *H*, 35 ml. pooled from two patients with typical infective hepatitis. Serum *N* was negative and *H* was strongly positive to all the tests used.

Preliminary experiments were also conducted on two other pooled sera with very similar results, but these will not be reported here as in these earlier separations the standard electrophoretic technique was combined with either precipitation or concentration by evaporation from the frozen state, such procedures being suspect in that they may cause modifications of the serum proteins or their complexes.

METHODS

Electrophoresis

The yields, particularly of α- and β-globulins, obtainable by the discontinuous electrophoretic technique of Tiselius (1938) (see, for example, Blix, Tiselius & Svensson, 1941) are so limited as to make a semi-continuous technique greatly preferable. Svensson (1942, 1946) has described a preparative apparatus which allows sampling and refilling to be carried out at will during the separation. For our purposes, Svensson's elaborate arrangement did not seem necessary, and a very simple modification of the standard Tiselius assembly employing the same principles was found adequate (Fig. 1).

The standard long cell (Longsworth, Cannan & MacInnes, 1940) (vol. of one limb + bottom section approx. 10 ml.) was connected to open electrode vessels (vol. 1700 ml.) by special junction pieces, *J*, allowing two withdrawal capillaries, C_U, C_L, to be permanently supported in each limb of the U-tube by rubber bungs, *B*. It was found to be a considerable advantage if the glass tubes (internal diam. 2·5 mm.) from which the capillaries were drawn, were gripped by the rubber bungs sufficiently loosely to allow the height of the capillary endings in the U-tube limbs to be

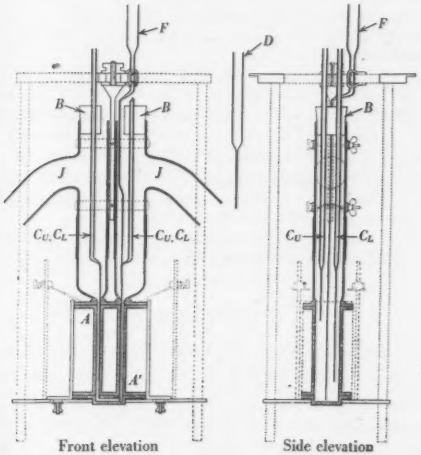

Fig. 1. Electrophoresis U-tube arranged for preparative separations; *F*, filling tube; *D*, compensator; *B*, rubber bungs; *J*, junction pieces; C_U, upper sampling capillaries; C_L, lower sampling capillaries; *A*, *A'*, boundary starting positions. Electrode vessels not shown.

adjusted during the progress of the separation. A wider glass tube, *F*, also drawn out into a capillary, and supported by a clip on the frame so that its tip touched the floor of the cell, served as a filling tube. The apparatus was filled with buffer solution, and brought to 0–1° in a water thermostat, before cooled saturated KCl solution was carefully led into each electrode vessel to more than cover the Ag-AgCl electrodes.

About 35 ml. of serum were dialyzed in the cold, over a period of 3 days or more, against several changes of potassium phosphate buffer, ionic strength 0·2, pH 8·0. (The pH of this buffer lies within the stability range of the serum proteins (Svedberg & Sjögren, 1930); cf. the finding of Moore *et al.* (1945) that γ-globulin separated in diethylbarbiturate buffer at pH 8·5 does not flocculate cephalin-cholesterol emulsions. With the lower conductivity, however, it permits a more rapid separation than does the pH 7·4 phosphate-NaCl buffer used by the above authors, and is also preferable in that it involves virtually no losses by spontaneous flocculation of either sera or fractions.) The dialyzed sample was clarified by centrifugation and about 12 ml. introduced into the cell very slowly through the filling tube *F*. (The optical density of the icteric serum necessitated some prior dilution.) The lower withdrawal capillary, C_L, of each limb was used to sharpen the protein boundary as it approached the capillary tip in the U-tube. Very sharp boundaries were obtained in this way, and were brought to positions *A* and *A'* for the start of their migration by a slow feed of buffer solution into the appropriate electrode vessel from a removable glass tube drawn out into a capillary *D*. The current density in the U-tube was kept sufficiently low to avoid convectional disturbance of the boundaries, but was so chosen as to give about the maximum extent of migration allowed by the dimensions of the U-tube at convenient sampling times (9–19 ma./cm.²; sampling twice daily).

Sampling was controlled by observation of the boundaries by the optical method of Philpot (1938). Albumin and albumin + α-globulin were withdrawn in turn from the centre of their respective boundaries by the upper capillary C_U of the ascending limb, γ- and β + γ-globulins being recovered from the descending side. Sampling was accompanied and controlled by compensation with *D* and by the introduction of further material into the bottom of the U-tube, the lower boundaries in each limb being at the same time fused by removal, via the lower capillaries, of the material composing them. These operations were so ordered as to leave the system of boundaries in a position suitable for further migration. The multicomponent mixtures removed by the lower capillaries were combined, reconcentrated by pressure dialysis, centrifuged, and used to refill the U-tube when the first stock of filling solution had become exhausted. Advantage was taken of several such refillings during the course of the separation to reverse the polarity of the electrodes, which involved no great inconvenience, since refilling with a fresh stock solution in itself entailed the formation of a new set of boundaries. Only two or three reversals were required over a period of 10 days or more continuous running. Recovery of unwanted fractions and their return to the U-tube was continued until sufficient quantities of albumin + α-globulin, and β + γ-globulin fractions had been obtained. These were then separated in the same way.

Figs. 2 and 3 are the electrophoretic diagrams at equivalent migration times of the original sera and the fractions albumin and γ-globulin obtained from them, indicating the degree of purity of the latter. Insufficient α- and β-globulin was obtained to submit them to electrophoretic analysis, but there is no reason to doubt that they were of a similar degree of homogeneity, except that in the case of the hepatitis serum the α-globulin was replaced by a boundary migrating so close to the β-globulin that it was only possible to isolate them together as one fraction, designated arbitrarily as (α + β)-globulin in the text. The overall yield of albumin and γ-globulin from serum *H* amounted to 21 and 27% respectively of the quantities estimated from the diagrams to be present in the original serum.

Throughout the processing the material was maintained at 0–4°, except for short operations such as centrifugation, and the sera and fractions were sterilized by filtration through 0·7μ pore Gradocol membranes (Elford, 1931) at various stages of the separation.

For the flocculation tests, the fractions were concentrated by pressure dialysis, and the buffer salt concentration reduced by dialysis against the volume of saline required to give at equilibration an ionic strength 0·09 in

NaCl, 0·01 in potassium phosphates. The final protein concentration was determined by the micro-Kjeldahl method, using a factor of 6·25 to convert nitrogen to protein.

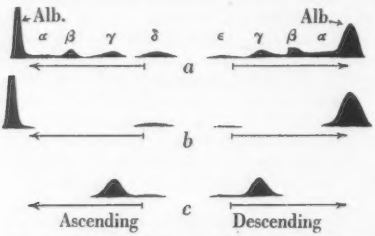

Fig. 2. Electrophoretic patterns of (*a*) normal serum *N* (by average of the two patterns 61·5% albumin, 9·7% α-globulin, 13·9% β-globulin, 14·9% γ-globulin); (*b*) isolated albumin fraction; (*c*) isolated γ-globulin fraction. K_2HPO_4 buffer, μ 0·2, pH 8·0. Refractive increment due to protein = 0·0030 for the serum, proportionately less for the fractions. Potential gradient = 3·21 V./cm., time 28,000 sec., electrode vessels open.

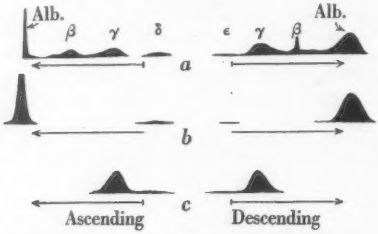

Fig. 3. Electrophoretic patterns of (*a*) hepatitis serum *H* (by average of the two patterns 54·0% albumin, 19·5% $\beta_1 + \beta$-globulin, 26·5% γ-globulin); (*b*) isolated albumin fraction; (*c*) isolated γ-globulin fraction. K_2HPO_4 buffer, μ 0·2, pH 8·0. Refractive increment due to protein = 0·0030 for the serum, proportionately less for the fractions. Potential gradient = 3·21 V./cm., time 28,000 sec., electrode vessels open.

Flocculation tests

The methods employed were:

(1) Cephalin-cholesterol test. Hanger (1939) 'Difco' antigen was used (Baird and Tatlock).

(2) Serum colloidal gold reaction. Earlier experiments were done with an oxalate sol at pH 7·8 (Maclagan, 1944*b*) and later ones with a citrate sol at pH 7·7 (Maclagan, 1946). These two methods are equivalent in sensitivity.

(3) Cerebro-spinal fluid colloidal gold reaction at pH 6·6 (Maclagan, 1946).

(4) Thymol turbidity test (Maclagan, 1944*a*). The photoelectric method of Shank & Hoagland (1946) was also tried for reading the turbidities, but the results appeared to be somewhat lower and this method was not pursued.

(5) Thymol flocculation test. This addition to the thymol turbidity test consists merely in leaving the mixture to stand overnight, after the turbidity has been read, as proposed by Neefe (1946). The amount of flocculation is graded as negative, 1, 2, 3 or 4+.

(6) Thymol-cephalin test. This was identical with the thymol turbidity test except that one drop of 1% cephalin in ether was added to the protein solution before the thymol reagent. The cephalin was prepared from sheep brain by the method of Hanger (1939). As explained below, this addition is not recommended for tests on whole serum.

(7) Takata-Ara reaction. Crane's modification as described by Chasnoff & Solomon (1938).

In all cases the actual volume of protein solution had to be varied up to a maximum of 0·5 ml.

RESULTS

The results obtained with single fractions are shown in Table 1, the only positive findings being that both γ fractions reacted with all three reagents, and the hepatitis (α + β) fraction reacted quite strongly with the cephalin-cholesterol reagent. Hepatitis γ-globulin was ten times as effective as normal γ-globulin against cephalin-cholesterol, and three times as effective against the gold reagent. The turbidity produced by the γ-globulin with the thymol reagent was however very small, being only about 1 unit/mg. in a volume of 3 ml. This result suggested the possibility that the serum phospholipids, which were known to be present in the precipitate obtained when the thymol test is done on whole serum, were also essential in the reaction and these might well be absent or diminished in pure γ fractions, being lost either during electrophoresis or during the preliminary dialysis.

Table 1. *Effect of single fractions*

(The weights given in the case of positive reactors are the amounts required to produce 1 unit of turbidity in the case of the thymol tests, and the least amounts required for complete precipitation in the other tests.)

Fraction	Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
Normal albumin up to 5 mg.	0	0	0	0
Normal α-globulin up to 0·4 mg.	0	0	0	0
Normal β-globulin up to 0·4 mg.	0	0	0	0
Normal γ-globulin	+ (1 mg.)	+ (0·1 mg.)	+ (0·075 mg.)	+ (0·5 mg.)
Hepatitis albumin up to 5 mg.	0	0	0	0
Hepatitis (α + β)-globulin up to 1·2 mg.	0	0	0	+ (0·06 mg.)
Hepatitis γ-globulin	+ (0·8 mg.)	+ (0·1 mg.)	+ (0·025 mg.)	+ (0·05 mg.)

The addition of various lipids to the fractions before the thymol reagent was therefore tried, and it was found that either lecithin or cephalin had the effect of giving a much increased turbidity in the case of γ fractions, amounting to 10 units/mg. for normal and 12 units/mg. for hepatitis γ-globulin. This addition had no effect on the negative results with albumin and α and β fractions. The technique finally adopted was that given under 'thymol cephalin test' above. It would appear from this that the serum phospholipids are an essential ingredient of the thymol reaction, and that results with electrophoretic fractions may be misleading unless the phospholipids are replaced.

The results with pairs of fractions from normal serum are shown in Tables 2 and 3. The principal points shown are:

(1) Normal albumin inhibits thymol-cephalin, gold and cephalin-cholesterol tests with some quantitative differences.

Table 2. *Effect of normal albumin on action of normal γ-globulin*

Note to Tables 2–6

The ratios given show the smallest amounts required to produce complete inhibition of one part of γ-globulin, the amounts of γ-globulin used being 0·1 mg. for the gold and cephalin-cholesterol tests and 0·6 mg. for thymol-cephalin tests. The experiments were done by a serial dilution technique with a dilution factor of $\frac{1}{2}$, so that an inhibition ratio of e.g. 10 : 1 may be taken to indicate that a 5 : 1 ratio failed to produce complete inhibition.

Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
0	Inhibited 4 : 1	Inhibited 10 : 1	Inhibited 100 : 1

Table 3. *Effect of normal α- and β-globulin on action of normal γ-globulin*

Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
0	10% increase of sensitivity	Inhibited $0{\cdot}5\alpha : 1\gamma$ $1\beta : 1\gamma$	Sensitivity unaltered but flocculation more rapid

(2) Normal α- and β-globulins are about ten times more effective than albumin in inhibiting the gold reaction but they do not inhibit the other two at all, producing instead a slight increase of sensitivity with thymol-cephalin and more rapid flocculation with cephalin-cholesterol.

Tables 4, 5 and 6 give the results obtained with pairs of fractions from hepatitis serum. They show:

(1) Hepatitis albumin still inhibits the gold and cephalin-cholesterol tests but fails to inhibit thymol-cephalin and slightly augments thymol alone.

Table 4. *Effect of hepatitis albumin on action of hepatitis γ-globulin*

Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
Sensitivity increased threefold	0	Inhibition 40 : 1	Inhibition 40 : 1

Table 5. *Effect of hepatitis $(\alpha+\beta)$-globulin on action of hepatitis γ-globulin*

Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
Sensitivity increased tenfold	10% increase of sensitivity	Inhibition 1·5 : 1	Sensitivity increased (active alone)

Table 6. *Effect of hepatitis albumin on action of hepatitis $(\alpha+\beta)$-globulin*

Cephalin-cholesterol:	Inhibition 40 : 1

(2) Hepatitis $(\alpha+\beta)$-globulin behaves like normal α and β against thymol-cephalin and gold, but markedly increases the sensitivity of thymol alone and cephalin-cholesterol. This fraction is of course active by itself against cephalin-cholesterol and possibly influences thymol alone by virtue of its lipid content.

(3) With cephalin-cholesterol, hepatitis albumin inhibits $(\alpha+\beta)$ fractions in about the same ratio as that required for inhibition of γ-globulin.

Gold reaction at pH 6·6. Some experiments were also conducted with colloidal gold in the buffer of pH 6·6, ionic strength 0·067, recommended for cerebrospinal fluid tests (Maclagan, 1946). The principal differences noted were a much increased sensitivity, 2 μg. of normal γ-globulin being sufficient for complete precipitation. At this pH there was little difference between normal and hepatitis γ-globulin, the latter requiring 2·5 μg. for complete precipitation. Normal and hepatitis albumin were both inhibitory in the proportion of 40 : 1, and hepatitis $(\alpha+\beta)$-globulin was inhibitory at 3 : 1. In these inhibitory experiments both 5 and 50 μg. of γ-globulin were used, the ratios of inhibitor required being the same with the two amounts.

Takata-Ara reaction. Only the hepatitis fractions were available for this test. γ-Globulin and $(\alpha+\beta)$ fractions were active and both completely flocculated the reagent down to 0·25 mg. Albumin inhibited the γ-globulin in the ratio of 2·5 : 1, and also inhibited the $(\alpha+\beta)$-globulin in the ratio of 0·67 : 1 (0·3 mg. of γ-globulin used).

Cephalin and thymol turbidity test on serum. In view of the effects shown above it was obviously important to try the effect of added cephalin on the thymol turbidity test as carried out on serum. A number of normal and pathological sera were therefore tested with and without added cephalin, but no

appreciable increase of turbidity was noted. It would appear therefore that in whole serum the native phospholipids are usually sufficient in amount to give a maximal turbidity with the thymol reagent, and nothing is to be gained by the addition of cephalin.

Thymol flocculation test. It was originally noted by one of us (Maclagan, 1944*b*) that in the case of sera giving positive thymol turbidity tests the mixture usually flocculated on standing overnight, but it did not at that time appear that a test based on this observation would present any advantage over the quicker measurement of turbidity. However, Neefe (1946) and Neefe & Rheinhold (1946) have since made extensive observations on this 'thymol flocculation test' and regard it as an important addition to the thymol turbidity test. Particular note was therefore made in this study as to the occurrence of flocculation, with the rather startling result that the hepatitis γ fractions either alone or in combination always produced flocculation whereas the normal γ fractions did not. This was true with thymol alone as well as with the thymol-cephalin test. It appears therefore that the observation of flocculation does give extra information and shows a qualitative difference between normal and hepatitis γ-globulin fractions. The thymol flocculation test was not inhibited by any of the other hepatitis fractions, but it was not possible to test normal fractions for inhibitory power as they had all been used up before the hepatitis γ fraction became available.

DISCUSSION

Although the results presented are based principally on only two sera, we feel that they are sufficiently detailed to serve as a basis for the interpretation of the tests. The principal reason for undertaking this work was to elucidate the mechanism of the thymol turbidity test, and the results show that this test is in the first place dependent upon the presence of native serum phospholipids, as might have been anticipated from the character of the precipitate, which had previously been shown to contain an average of 0·33 % P, corresponding to 8·0 % of phospholipid (Maclagan, 1944*a*). Results with electrophoretic fractions are therefore liable to be misleading since the lipids are associated in high proportion only with the α- and β-globulins, and are particularly deficient in isolated γ-globulin fractions (Blix, Tiselius & Svensson, 1941). If, however, cephalin is added to the fractions results of the type anticipated from analogies with the related tests are obtained. These are given above under thymol-cephalin reactions, the principal features being:

(*a*) γ-Globulin is the only fraction active alone (activity 10–12 units/mg. in a volume of 3 ml.).

(*b*) Normal albumin inhibits the reaction in the ratio of 4:1, thus accounting for the negative reactions of normal sera.

(*c*) Hepatitis albumin and both normal and hepatitis α- and β-globulin have little effect.

(*d*) Only the turbidity developed by hepatitis γ-globulin proceeded to flocculation on standing.

Since the addition of cephalin to whole serum does not alter the results obtained, we consider that the test as originally described depends upon the mechanism outlined above. In any case there is no advantage in adding cephalin in the case of whole serum.

These results appear to contradict the work of Recant *et al.* (1945) who were unable to demonstrate any thymol turbidity with γ fractions separated electrophoretically at pH 7·4, either with or without added lipoids. These authors, however, do not give the details of their experiments.

The results obtained with colloidal gold show two important differences from those of previous workers: first, the considerably greater flocculating power of hepatitis γ-globulin as compared with normal γ at pH 7·8, and secondly, the powerful inhibitory effect of both normal and hepatitis α and β fractions. On the basis of the figures given above it is possible to formulate two equations expressing the excess flocculating activity (FA), in terms of γ-globulin, at pH 7·8 of the normal and hepatitis serum respectively. These are:

(1) $FA = \gamma - 2{\cdot}0\,\alpha - \beta - 0{\cdot}1$ Alb. for normal serum N,

(2) $FA = \gamma - 0{\cdot}67\,(\alpha + \beta) - 0{\cdot}025$ Alb. for hepatitis serum H.

The terms Alb., α, β and γ refer to the relative concentrations of the respective components expressed as percentages of the total serum protein. Although based on our nitrogen estimations, the approximate nature of these equations allow the use of uncorrected refractometric (electrophoretic) percentages. When applied to our whole sera they give the following values:

For the normal serum, $FA = -24{\cdot}5$,
For the hepatitis serum, $FA = +12$,

in qualitative accordance with the observed behaviour. Similarly all the normal sera analyzed by Gray & Barron (1943) give negative FA values by equation (1) and apart from one case of post-arsphenamine jaundice, a disease in which negative flocculation tests are particularly frequent (Maclagan, 1944*a*), four out of five of their hepatitis sera would be expected to have gold sol flocculating activity according to equation (2). It is of interest that most normal sera give negative values in (2) as well as in (1).

Our results with the cephalin-cholesterol test also differ from those of, for example, Moore *et al.* (1945) in showing a greater flocculating power of hepatitis as opposed to normal γ-globulin, and in the marked flocculating activity of hepatitis $(\alpha + \beta)$-globulin.

The latter has not previously been observed. The inhibitory ratio of 100:1 for normal albumin and γ-globulin does not account for the negative reaction of normal serum.

In comparing the various flocculation tests investigated (Table 7) it will be seen that there is a distinct family resemblance between them as γ-globulin is active in all of them and, so far as they have been tested, they are all inhibited by normal albumin. It is therefore not surprising that the tests have all proved useful as liver function tests and that the results on the whole run roughly parallel with each other. However, the failure of hepatitis albumin to inhibit the thymol reaction, and particularly the different functions of α- and β-globulin in the various tests, are sufficient to account for some individual sera reacting differently in different tests, as for example reported by Watson & Rappaport (1945). The thymol turbidity test appears to be the simplest in mechanism as it depends only upon the two fractions albumin and γ-globulin. It may therefore be described as a test of relative γ-globulin excess. The thymol flocculation test is a more qualitative procedure which is nevertheless of importance as it shows striking differences between normal and hepatitis γ-globulins. It is therefore evidently desirable to combine this test with the thymol turbidity test as proposed by Neefe (1946).

While the mechanism of these tests is not completely understood it is evident that they depend upon differences in chemical structure and not simply upon the net electric charge on the protein. This is shown by the fact that all the reagents are negatively charged colloids and are yet flocculated at reactions well above the isoelectric points of any of the serum proteins, so that the proteins must also be negatively charged under the conditions of the tests. The adsorption experiments of Elkes, Frazer, Schulman & Stewart (1945) suggest that this property, even if not peculiar to γ-globulin, is unusual in soluble proteins. The marked differences between the behaviour of normal and hepatitis fractions are not accompanied by gross electrophoretic differences. (Exact measurements of mobility were not however made during the course of this work.) The suggestion is that these flocculation tests can reveal differences in protein structure not shown by other physical methods, and may well lend themselves to wider application in protein chemistry. They have also the advantage of technical simplicity compared with, for example, serological tests.

Table 7. *Comparison of flocculation tests*

Test	Flocculator	Inhibitor	Normal-hepatitis differences
Thymol (cephalin) turbidity test	γ-Globulins	*N* albumin	*H* γ slightly more effective than *N*; *H* albumin not inhibitory
Thymol (cephalin) flocculation test	*H* γ-globulin only	None demonstrated (*N* fractions not tested)	*N* γ-globulin not effective
Gold at pH 7·8	γ-Globulins	Albumins, α- and β-globulins	*H* γ-globulin more effective than *N*
Gold at pH 6·6	γ-Globulins	Albumins, α- and β-globulins	None demonstrated
Cephalin-cholesterol	γ-Globulins; *H* ($\alpha+\beta$)-globulin	Albumins	*H* γ much more effective than *N*; *N* α and β not effective
Takata-Ara	*H* γ-globulin; *H* ($\alpha+\beta$)-globulin	*H* albumin	*N* fractions not tested

SUMMARY

1. A modified technique is described, suitable for the preparation of electrophoretically pure proteins from relatively small volumes of serum. Two human sera were separated, normal (*N*) and hepatitis (*H*).

2. The thymol turbidity test as carried out on serum depends partly upon the presence of native phospholipids. In tests on protein fractions with added cephalin γ-globulin acted as a precipitating agent; *N* albumin inhibited the reaction and *H* albumin did not; α and β fractions were without significant effect.

3. The thymol flocculation test, both with and without added cephalin, was positive with *H* γ-globulin, but was negative with *N* γ-globulin and with all other fractions. It was not inhibited by other *H* fractions (*N* fractions were not tested for inhibition).

4. Corresponding data are presented for the colloidal gold reaction, the cephalin-cholesterol test, and the Takata-Ara reaction. Two formulae have been obtained which qualitatively account for the behaviour of whole sera with the gold reagent.

5. The effect of α- and β-globulin was different in each test. Thus *H* ($\alpha+\beta$)-globulin flocculated the cephalin-cholesterol and Takata-Ara reagents while both *N* and *H* α- and β-globulins were powerful inhibitors of the gold reaction.

6. The possible value of these tests as indicators of differences in protein structure is discussed.

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POSTSCRIPT

While this paper was in the press there have come to our notice two further references to similar work. Cohen & Thompson (1947) describe experiments on the electrophoresis of sera before and after treatment with the thymol reagent, which they interpret as indicating that the β-globulin fraction is the active agent in the thymol test. They report only slightly higher concentrations of β-globulin in positive sera and therefore suggest the possibility of a qualitative change in this fraction. There are two important differences in technique which may account for the discrepancy between their conclusions and ours. First, Cohen & Thompson's (1947) electrophoresis was conducted at pH 8·6 and ours at pH 8·0. The analytical classification is thus not necessarily identical, for as has been noted above γ-globulin separated at pH 8·6 is devoid of the flocculating activity associated with γ-globulin separated at a lower pH (Moore *et al.* 1945). Secondly, the conditions of precipitation were not identical, as both the initial ionic strength and the final protein concentration would be much higher in Cohen & Thompson's (1947) experiments. They added only 2 vol. of thymol reagent to one of serum, and then dialyzed against a further 20–25 vol. of reagent, whereas the thymol test is performed by adding 60 vol. of reagent to one of serum. Their results would thus not necessarily apply to the thymol turbidity test as actually performed on serum.

Hanger (1946) has made a brief report on work which appears to be entirely in agreement with our own, in that he finds γ-globulin to be the active precipitating agent in the thymol test with phospholipids as an essential adjuvant.

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A Method for the Estimation of Creatinine

By J. A. BARCLAY and R. A. KENNEY
Department of Physiology, Medical School, Hospitals Centre, Birmingham

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It has always been realized that the alkaline picrate method (Folin, 1905) for the estimation of creatinine suffers from the defect of being non-specific (Hunter, 1928). A wide variety of substances react, among them glucose, formaldehyde, acetaldehyde and many creatinine-like nitrogenous compounds, some of which are likely to be present in biological fluids. 3:5-Dinitrobenzoic acid (Langley & Evans, 1936) appears to offer greater specificity but only at the expense of sensitivity. Barrett (1936) described a qualitative test for creatinine using a reagent based on Nessler's solution. Further study has shown that such a reagent could be made the basis of a quantitative method, the estimation being carried out nephelometrically.

METHODS

The preparation of the reagent. Barrett's solution is prepared by adding KI to a Nessler's solution; the effect of varying the amount of KI added was first investigated (Fig. 1). It will be seen that the addition of KI diminishes

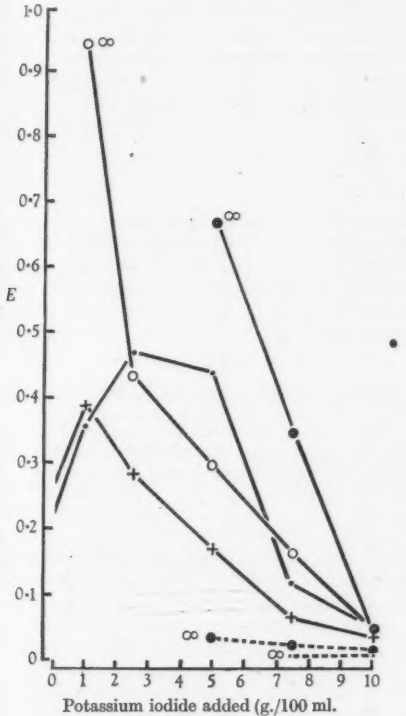

Fig. 1. The effect of added KI on the reaction of a Nessler's solution with creatinine and some biologically related substances. +——+ Creatinine, 1 mg./100 ml. solution; •—• creatine, 100 mg./100 ml. solution; ●——● hydantoin, 100 mg./100 ml. solution; ○——○ creatinine 1 mg./100 ml. + glucose 100 mg./100 ml. solution; ●- - -● guanidine 100 mg./100 ml. solution; --------- ammonia 1 mg./100 ml. solution; ∞ denotes the appearance of a dense precipitate.

the reaction of the Nessler's solution with ammonia, guanidine and hydantoin, and reduces the promoting effect of glucose on the creatinine reaction. The creatine and creatinine reactions reach a peak as KI is added and thereafter the reaction is reduced. Either the point of greatest sensitivity to creatinine or the point where the reaction with interfering substances is at a minimum may be taken for analysis of biological fluids. A compromise must be reached, and the addition of 7·5 g. of KI/100 ml. of Nessler's solution would appear to give a satisfactory reagent.

It was found, however, that Nessler's solution differed from batch to batch and that each required an individual adjustment with KI. It was found possible, however, to

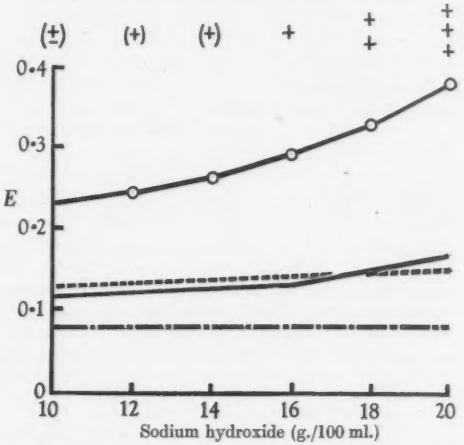

Fig. 2. The effect of alkalinity on the reaction of the artificial reagent containing 5% (w/v) KI. o—o hydantoin, 100 mg./100 ml.; - - - - - - glucose 100 mg./100 ml. + creatinine, mg./100 ml.; —— creatinine, 1 mg./100 ml.; —·—·— creatine 100 mg./100 ml. Reaction with ammonia (1% solution) ranging from + + + dense red ppt. to very slight ppt. (±).

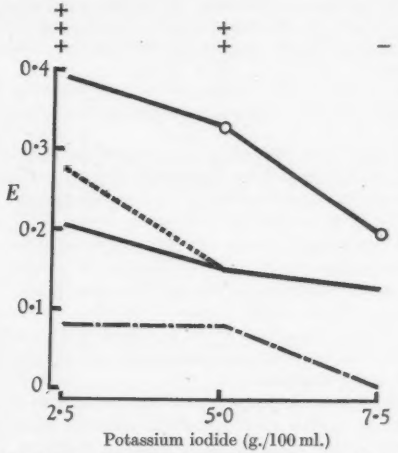

Fig. 3. Effect of varying KI concentration on the sensitivity of the 'artificial' reagent containing 18% NaOH: symbols as in Fig. 2.

prepare an 'artificial' solution which would react in the same fashion by the addition of KI to an alkaline solution of potassium mercuric iodide. In Fig. 2 is shown the effect of varying the alkalinity of such a solution, the KI concen-

Table 1. *Creatinine content of autoclaved rat tissues: comparison of Folin and nephelometric methods*

(All values expressed as mg./100 g. wet tissue.)

	Folin method				Nephelometric method			
Tissue	Before treatment with creatinine-destroying bacilli (*A*)	After treatment (*B*)	True creatinine (*A* − *B*)	Ratio $\frac{A}{(A-B)}$	Before treatment with creatinine-destroying bacilli (*A*)	After treatment (*B*)	True creatinine (*A* − *B*)	Ratio $\frac{A}{(A-B)}$
Liver	12·5	7·5	5·0	2·5	5·9	0·9	5·0	1·18
Kidney	60	31	29	2·07	34	6	28	1·21
Spleen	60	41	19	3·16	20	3	17	1·18
Muscle	580	200	380	1·53	400	60	340	1·17
Average	—	—	—	2·31	—	—	—	1·184

tration being fixed at 5% (w/v). It will be seen that increasing the strength of the alkali markedly increases the reaction towards ammonia, hydantoin and, to a lesser extent, to creatinine. At an NaOH concentration of 18% (w/v), glucose has no promoting effect on the reaction and so a solution of this alkalinity was chosen as the most promising for further study. In Fig. 3 is shown the effect of varying the KI concentration of a solution containing 18% (w/v) of NaOH. When the KI concentration is increased to 7·5% (w/v) creatine and ammonia cease to react and the hydantoin reaction is very reduced. This then was chosen as the optimal concentration of KI and the composition of the reagent becomes: 1 g. potassium mercuric iodide, 18 g. NaOH and 7·5 g. KI, all dissolved in water, made to 100 ml. and mixed.

In addition to creatine and ammonia, substances giving no reaction include guanidine, methyl guanidine, sarcosine and uric acid; the hydantoin reaction is so slight as to make this reagent almost specific for creatinine in biological fluids.

PROCEDURE

Protein-free solutions. The sample is diluted to contain less than 3 mg. creatinine/100 ml. and 3 ml. of this solution are added to 1 ml. of the reagent. The solutions are mixed and allowed to stand for 5 min. The precipitate which appears is very stable and remains dispersed for at least 30 min. It is estimated nephelometrically in a photoelectric absorptiometer, using a Chance O.B. 2 glass light filter. Standard solutions covering the range required (not exceeding 3 mg. creatinine/100 ml. of solution) are treated with each set of samples.

Blood and plasma samples. The sample is deproteinized by the zinc hydroxide method (Somogyi, 1930) or the tungstic acid method (Folin & Wu, 1919). The filtrate is then estimated as above, using standard solutions treated in the same manner as the samples.

Tissues. The tissue is finely minced and mixed with a known volume of water. The suspension is mixed by shaking, filtered and the resulting clear solution treated in the same manner as a plasma sample, the solution being adjusted by dilution to contain less than 3 mg. creatinine/100 ml. before estimation. For the estimation of total creatinine of a tissue the suspension of the material in water is autoclaved at 30 lb. pressure for 1 hr. and then treated as above.

RESULTS

Recovery of creatinine added to human plasma

In Fig. 4 are illustrated the results of a typical recovery experiment performed on a sample of human plasma to which creatinine has been added.

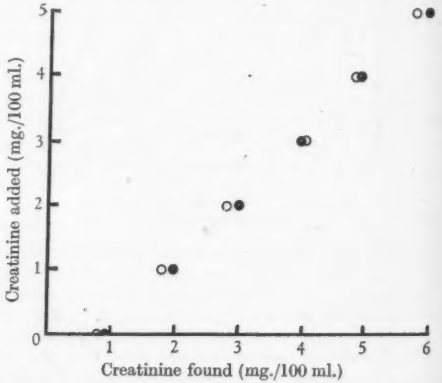

Fig. 4. Recovery of creatinine added to normal human plasma. • Samples deproteinized by the zinc hydroxide method (Somogyi, 1930); o samples deproteinized by the tungstic acid method (Folin & Wu, 1919).

The recovery obtained on the tungstic acid filtrates is slightly less satisfactory than that obtained on the zinc hydroxide filtrates and the former method gives a slightly lower value for the normal plasma creatinine level (zinc hydroxide filtrate 0·95 mg. creatinine/100 ml.; tungstic acid filtrate 0·87 mg./100 ml.). Similar results have been obtained on dog plasma.

The specificity of the reagent was compared with that of the Folin method by estimating the apparent creatinine content of autoclaved rat tissues before and after treatment with the specific creatinine-destroying bacillus N.C. of Miller & Dubos (1937). The analyses were made in both cases on zinc hydroxide filtrates of the tissue and performed against standards treated in the same fashion. The results show that the ratio of apparent to true creatinine is consistently smaller in the case of this method than in the Folin method. The results obtained for true creatinine agree well with those of Miller, Allison & Baker (1939).

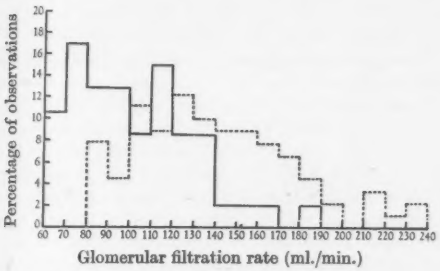

Fig. 5. Histograms of glomerular filtration rates in a group of normal subjects. Solid line, values determined by inulin method; dotted line, values determined by creatinine method.

Simultaneous estimations of creatinine in blood and urine have been performed and the renal clearance of endogenous creatinine compared with that obtained by the inulin method (Smith, 1943). In Fig. 5 are plotted histograms of creatinine and inulin clearance values determined on normal adult subjects. The mode of the creatinine values lies at a value of 136 ml./min. Whilst higher by 30 ml./min. than our own inulin clearances the value is identical with that obtained by Goldring & Chasis (1944).

DISCUSSION

We have approached the problem of the preparation of a suitable reagent for creatinine by two methods. We have first found the best conditions for specificity and sensitivity of the reagent and then compared the reagent so prepared with the Folin reagent. It can be seen from the figures that by suitable adjustment of NaOH and KI concentration, the reaction with substances which interfere with the Folin method can be eliminated or so diminished as to render negligible the error involved in applying this reaction to biological fluids. The very much reduced reaction with the chromogenic material of rat tissues confirms this observation.

We have then applied this reagent to the estimation of glomerular filtration rate since it was felt that if the reagent were sensitive to chromogenic material of either blood or urine, the clearance value so obtained would show a marked deviation from the normal figure, it being unlikely that the chromogenic substances would have the same renal clearance as creatinine. Two points can be seen from these results. The clearances obtained are very much lower than those obtained by workers (Rehberg, 1926) who estimated their creatinine by the Folin method. Secondly, the close agreement between the clearances herein quoted and those obtained by the inulin method suggests that we are dealing with a single substance and one which is treated in a very special way by the kidney. This offers support for the postulate of Ekehorn (1944) that creatinine provides a true measure of filtration rate. It will be seen that by the simultaneous use of two reagents prepared along these lines it is possible to estimate, for example, hydantoin or creatine in the presence of creatinine.

SUMMARY

1. A rapid method for the estimation of creatinine is described which has a greater specificity than the Folin method.

2. The extent of chromogenic interference of non-creatinine substances is illustrated by the estimation of apparent and true creatinine of autoclaved rat tissues.

3. When creatinine is estimated in blood and urine by this method the creatinine clearance gives a true measure of filtration rate.

Our thanks are due to Dr Rene Dubos for a strain of the N.C. bacteria.

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The Identification of Lower Peptides in Complex Mixtures

By R. CONSDEN, A. H. GORDON and A. J. P. MARTIN
The Wool Industries Research Association, Torridon, Headingley, Leeds 6

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The mixture of amino-acids and peptides, which is obtained when proteins are only partially hydrolyzed by acids, is so complex that no single method can be expected to achieve the separation and identification of more than a few of the components present. The successive use of methods effecting separations along different lines is required: a scheme of separation is described here which, it is believed, can lead to the identification of a large proportion of the dipeptide species present in partial acid hydrolysates of proteins (cf. Martin, 1946). The scheme includes the use of two previously published methods, ionophoresis in silica jelly (Consden, Gordon & Martin, 1946*a*) and partition chromatography on paper (Consden, Gordon & Martin, 1944), a method of freeing solutions of amino-acids and peptides from salts, a micro-technique for the hydrolysis, and a method for the deamination and subsequent hydrolysis of material obtained from a paper chromatogram.

The partial hydrolysate is first subjected to ionophoresis, at a pH near 6. In this way, a division into about a dozen distinct fractions differing in their mobility in an electric field, is obtained. Method B (Consden *et al.* 1946*a*), in which the silica jelly is set beneath a glass lid, has been adopted because it gives the most complete separation. However, since the jelly is made from sodium silicate and an acid, and replacement by another electrolyte is impracticable, the finally separated fractions are contaminated, not only with silica, but with some sodium salt. Consden *et al.* (1946*a*) discuss various methods of removing salts from amino-acid solutions but none were suitable for the removal of sodium acetate, which has been found so far to be the most suitable electrolyte for a pH near 6. The removal of sodium acetate has been effected in two stages. First, acetate was replaced by sulphate by steam distillation *in vacuo* of a solution acidified with sulphuric acid. Secondly, sodium sulphate and sulphuric acid were removed by a 'desalting apparatus'. This 'desalting apparatus', a preliminary account of which has been communicated (Consden, Gordon & Martin, 1945), employs electrolysis between a carbon anode, enclosed in a cellophan membrane containing dilute sulphuric acid, and a circulating mercury cathode, washed with water, for the removal of strong electrolytes. The problem of removing a given salt is thus changed to the easier one of converting one salt into another, and the alkali metals are no longer troublesome. The field of use of the desalting apparatus thus probably greatly exceeds the use here described. The material obtained from the desalting apparatus is, after evaporation, ready for further fractionation by partition chromatography (Consden *et al.* 1944) or by other methods.

These paper chromatograms, which may be one- or two-dimensional, are carried out in duplicates or in larger sets; one is treated with ninhydrin and is used as a guide to the cutting of the other. The material washed from the cuts may be hydrolyzed or first deaminated by treatment with nitrous fumes and then hydrolyzed. The hydrolysis products are again subjected to chromatography for the identification of the constituent amino-acids. If the cut contains only a single dipeptide, its identity (apart from stereoisomerism) is shown in this way, since of the two amino-acids liberated by hydrolysis, that with the free amino group is destroyed after the deamination treatment. When, however, the cut contains several dipeptides or a heterogeneous mixture of peptides the interpretation of the result is often impossible. Even when only two amino-acids are present, a tripeptide with two residues of the same amino-acid cannot easily be excluded, since the depth of colour given by the ninhydrin is not an accurate measure of the amount of amino-acid present. A reasonably accurate method, applicable to the quantities involved, would be of the greatest assistance. The method of Martin & Mittelmann (to be published) may fulfil these requirements.

Because the R_F values of peptides are more sensitive than those of the amino-acids to changes of pH and to other unknown factors, it is desirable, where possible, to run mixed chromatograms with authentic samples to confirm the results of the analysis described above. For purposes of comparison the R_F values of some synthetic peptides are given in Table 1. In the absence of such confirmation, there is a real danger that with substances containing peptides as part of the molecule only, the other components will be overlooked, since the colour reaction with ninhydrin is often the only guide. Partridge (1946) has, however, been able to demonstrate the presence of reducing sugars by the use of a silver reagent and perhaps later other

Table 1. *R_F values of some peptides* in various solvents on Whatman no. 4 paper*

Solvent addition	Phenol 0·1% cupron	Phenol coal-gas 0·3% NH_3†	*s*-Collidine	*n*-Butanol 0·1% cupron	Benzyl alcohol 0·1% cupron	*m*-Cresol 0·1% cupron	*m*-Cresol 0·1% cupron 3% NH_3†
Glycylglycine	0·53	0·57	0·28	0·01	0·00	0·10	0·18
Glycyl-*dl*-alanine	0·64	0·63	0·32	0·03	0·01	0·20	0·29
Glycyl-*dl*-valine	0·78	0·74	0·47	0·09	0·02	0·37	0·51
Glycyl-*dl*-leucine	0·87	0·79	0·53	0·24	0·05	0·59	0·66
Glycyl-*l*-proline	0·77	0·69	0·34	0·05	0·03	0·68	0·69
Glycyl-*l*-hydroxyproline	0·57	0·59	0·28	0·02	0·01	0·49	0·25
Glycyl-*l*-phenylalanine	0·78	0·70	0·79	0·18	0·08	0·65	0·72
Glycyl-*l*-tryptophan	—	0·66	0·85	0·29	—	0·44	0·63
Glycylglycylglycine	0·58	0·59	0·32	0·01	0·02	0·08	0·19
Glycylglycyl-*l*-leucylglycine	0·86	0·75	0·71	0·11	0·04	0·67	0·67
dl-Alanylglycine	0·68	0·65	0·32	0·03	0·01	0·21	0·29
l-Alanylglycylglycine	0·67	0·63	0·46	0·01	0·00	0·19	0·29
dl-Valylglycine	0·83	0·73	0·53	0·11	0·04	0·48	0·48
dl-Leucylglycine	0·86	0·78	0·61	0·23	0·08	0·63	0·62
dl-Leucyl-*dl*-leucine	0·95	0·84	0·89	0·65	0·30	0·87	0·85
l-Leucyl-*l*-tryptophan	0·92	0·83	0·95	0·60	0·34	0·87	0·83
l-Leucylglycylglycine	0·88	0·75	0·55	0·11	0·04	0·61	0·60
l-Prolylglycine	0·86	0·68	0·49	0·03	0·03	0·67	0·55
l-Tyrosylglycine	0·59	0·59	0·95	0·09	0·03	0·27	0·33

Peptides in which glycine carries a free amino group, on heating with ninhydrin give, first, a yellow colour, then, grey and finally purple. All the other peptides in the table give a purple colour, except prolylglycine, which is yellow at first, then orange and finally, grey. Diketopiperazines do not give colours with ninhydrin.

* For the R_F values of peptides related to Gramicidin S, see Consden, Gordon, Martin & Synge (1947).

† The % figure refers to the strength of the NH_3 solution present in the tray at the bottom of the chamber.

reagents will allow a large variety of substances to be detected. In many cases, however, evidence of the existence of a non-amino-acid part of the molecule is afforded by an apparently inexplicable ionophoretic and chromatographic behaviour.

EXPERIMENTAL

Removal of silica. The silica jelly, cut from the ionophoresis trough, is reduced to a smooth paste by stirring in a beaker. Water (2 vol.) is added and the suspension is allowed to stand, with occasional stirring, for 2 hr. It is filtered on a Buchner funnel and washed with a further 2 vol. of water.

Removal of acetate ions and acetic acid. After removal of the silica, the filtrate and washings are acidified with 2N-H_2SO_4 until red to thymol blue (pH 2). Acetic acid is then removed by steam distillation under reduced pressure. The apparatus consists of a train of three round-bottomed flasks. The first flask of capacity 1 l. and fitted with a capillary, is immersed in a water bath at 50–60°. The steam is conducted to the bottom of the next flask (500 ml.) which contains the sample (50–100 ml.) and has a Claisen top and a suitable trap to diminish entrainment. The third flask (1 l.) acts as a condenser and is connected to a water pump.

The temperature of the Claisen flask is not allowed to rise above 40° and distillation is carried on until the volume is reduced to 30 ml. When about 500 ml. has been distilled, the condensate is washed out and a further 100 ml. of condensate is titrated against N-NaOH. If less than two drops are required, the removal of acetic acid is considered adequate. The sample is now ready to be transferred to the desalting apparatus, the sample and washings of the Claisen flask together forming a convenient volume.

Desalting apparatus

The apparatus (Fig. 1) consists of a Perspex tray, through which mercury is circulated by a water lift pump. A baffle is placed at the top of the exit tube

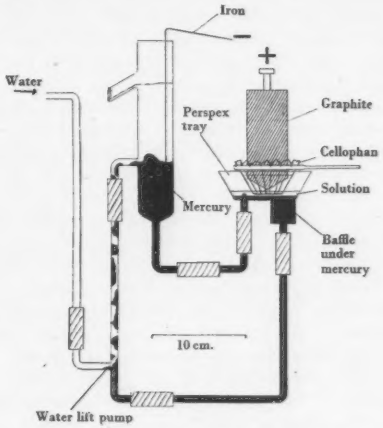

Fig. 1. Diagram of desalting apparatus.

of the tray to prevent the formation of whirlpools in the mercury. An iron cathode dips into the mercury in the pump. The solution to be desalted floats on the mercury. Into this dips a thick cellophan membrane containing 0·1N-H_2SO_4 in which is

immersed a graphite anode. The membrane permits the passage of anions of strong electrolytes to the anode, hydrogen ions passing in the reverse direction. By reason of this high H^+ concentration within the membrane, anions of weak acids lose their charge. Further, since the electro-endosmotic flow is towards the cathode, the diffusion of uncharged molecules into the anode compartment is practically negligible. The amino-acids, even the dicarboxylic amino-acids, acquire a net positive charge within the membrane and are thus less likely than uncharged molecules to reach the anode compartment.

The mercury cathode discharges hydrogen ions and other inorganic cations, hydrogen being liberated and the metals dissolve in the mercury. The alkali metals and earths are washed out of the mercury by the pump water. At high pH sodium, but at low pH hydrogen is preferentially discharged. Thus, if an attempt be made to remove sodium acetate, sodium ions are at first rapidly discharged, but as the amount of free acetic acid increases, the falling pH results in an increased hydrogen evolution, and decreased sodium removal, until at a pH between 3 and 4 practically no more sodium is eliminated. With sodium borate or sodium phenoxide, the sodium (but not the weak acid) can be almost entirely removed. Consider the removal of a sodium sulphate-sulphuric acid mixture. The rate of removal of sulphate ions is governed by the transport number of the sulphate ion in the membrane, and if the pH is low, hydrogen ions will be discharged faster than they come from the anode, causing the pH to rise. Conversely, if the pH is high initially, sodium ions will be removed preferentially and hydrogen ions will arrive from the anode faster than they are discharged, causing a fall of pH. Thus, the pH will attain a value at which the rate of removal of sulphate and sodium ions is identical. As the current continues to pass, the concentration of sodium sulphate drops until, finally, a very dilute solution of sulphuric acid, remains. The concentration of this acid depends on the current density and thickness of the membrane, since, in the absence of diffusion and electro-endosmosis, the concentration would drop to zero. Thus to obtain a very dilute solution, it is necessary to have a high potential between the electrodes, a thick membrane and a small area of contact between the membrane and the liquid.

As the concentration of the solution falls, the resistance of the apparatus rises and the current, falling to a steady small value, can be used as an indication of the end-point. Another indication of the progress of the removal of alkali ions may be obtained by testing the water issuing from the pump with indicators. As explained above, if a weak acid is present, all the salt cannot be removed, and the current becomes steady at a higher value. Ammonia can also be removed in this apparatus, but owing to the decomposition of the ammonium amalgam, the mercury becomes coated with soft lumps which have to be broken up and the current may have to be rather low in order to avoid choking up of the mercury-circulation system. When halides are removed, it is desirable to employ two concentric anode membranes, each containing initially sulphuric acid. In this way the amount of halogen released at the anode is greatly reduced and the amount of free halogen diffusing back to the solution being desalted is negligible.

At the beginning of the desalting, the voltage may be as low as 20 V. but is raised at the end to 300 V., unless weak acids are present. The current at first may rise to 2 amp. and fall at the finish to 50 ma. The circulating mercury effectively cools the solution to be desalted, and a maximum of about 100 W. can be dissipated without excessive rise of temperature. The current efficiency (ratio of equivalents removed to faradays passed) for the removal of salts depends on the nature of the salt to be removed. Thus, with NaOH, where removal of sodium is the limiting factor, the efficiency is about 80–85 %. With Na_2SO_4, where removal of SO_4^{--} is the limiting factor, the efficiency is 10–15 %; in other words, the efficiency is approximately equal to the transport number of SO_4^{--} in the membrane, the other ion being the hydrogen ion.

Recovery of glycine and glutamic acid from the desalting apparatus. To test whether amino-acids are lost either by passage into the anode solution, or by being carried away in droplet form by the mercury, known amounts of glycine and glutamic acid were desalted and then estimated by the copper titration method of Pope & Stevens (1939).

(1) 103 mg. glutamic acid dissolved in 50 ml. of 0·08N-NaOH were desalted (15 min. minimum current 0·4 amp.; starting voltage 50 V., final voltage 150 V.). The solution was pipetted off; the tray was washed out three times and the solution and washings were made up to volume. Titration showed 101 mg. glutamic acid (i.e. 98 % recovery). (2) 101 mg. glycine dissolved in 25 ml. 0·149N-NaOH were desalted, washed out, made up to volume and titrated. Recovery 101·3 mg. (100 %). (3) 101 mg. glycine dissolved in 25 ml. 0·08M-Na_2SO_4 were desalted, washed out, made up to volume and titrated. Recovery 98·2 mg. (97 %). Gas and liquid were visible in the tubes circulating the mercury. This liquid must be picked up as small drops of water in the pump since it did not become coloured when a strong solution of fuchsine was floated on the mercury.

One-dimensional chromatographic analysis

The solution, after desalting, is transferred to a 1 l. Claisen flask together with three 10 ml. washings, by means of a pipette. After evaporation *in vacuo*, below 40°, the sample is transferred to a 25 ml. conical flask, again with a pipette and evaporated to dryness. A known small volume of water is now added and a few μl. of the solution run on a paper chromatogram. After development and colouring with ninhydrin, the complexity of the mixture

can be gauged and a decision taken as to whether a one-dimensional or a two-dimensional chromatogram is needed. An estimate of the size of the measured sample required for further work can also be made.

If it has been decided that chromatography in one dimension will give sufficient separation, a strip of paper is prepared wide enough to have a number of spots (2–8) close together (6 mm. apart) and one spot separated from the others by 2 cm. or more. The number of spots taken should be enough to provide at least 10 μg. of each peptide for each solvent in which the amino-acids are to be run after hydrolysis. Several spots, rather than a single spot made with a larger amount of material, are used in order to avoid local overloading and each spot should contain the same amount of material, so that conditions should be as similar as possible.

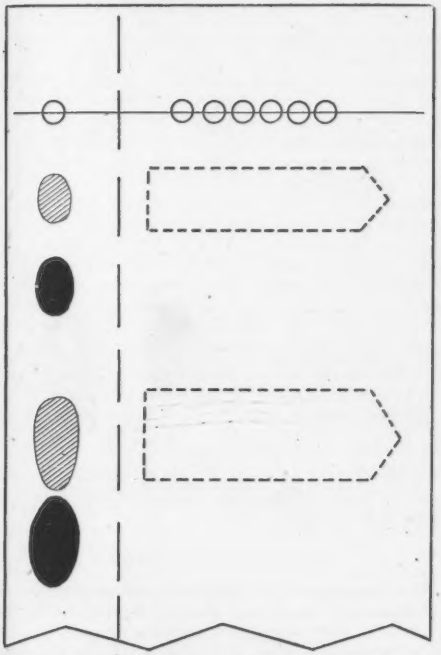

Fig. 2. Diagram showing method of cutting of a one-dimensional chromatogram. The test solution is applied along the top line (open circles). After development of the chromatogram, the left side of the sheet is cut off (broken line) and treated with ninhydrin to show amino-acid spots (black) and peptide spots (shaded). Using this as a guide, the remainder of the sheet is cut as shown by the dotted lines.

After the strip has been developed and dried, the chromatogram formed from the single spot, is cut off and treated with ninhydrin. This is then used as a guide for cutting up the rest of the strip. The 'cuts' are then trimmed at one end to a blunt point (Fig. 2). A trough similar to those used for the chromatograms is now provided with a strip of clean filter-paper running the full length of the trough and wide enough just to overlap the side bar. The trough is filled with water. To wash one of the 'cuts', the end opposite the point is held against the wet paper until about half an inch of the end of the 'cut' is wet. One-quarter inch of the wet end is then allowed to overlap the edge of the wet strip. After a few seconds a film of water between the papers anchors the 'cut' firmly in position. The water creeps slowly down the paper carrying the amino-acids, peptides, etc. with it. A thick walled capillary tube of 1 mm. bore and about 12 cm. long, with its ends drawn out is now adjusted so that one end touches the point of the cut, while the other end is slightly lower (Fig. 3). In the course of about 1 hr. the tube becomes filled, and if the cut had an area of not more than 10 sq.cm., washing is usually complete, as shown by testing the dried

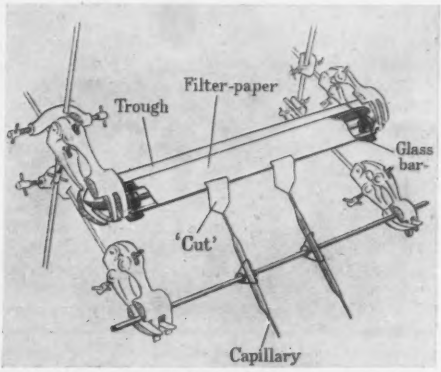

Fig. 3. Diagram of apparatus for washing material off 'cuts' from chromatograms (see Figs. 2 and 5*b*) into capillary tubes.

paper with ninhydrin. It is very important that the organic solvent should be thoroughly removed by drying from the chromatogram before the washing. If not, the water may run down irregularly and washing will be incomplete with this volume. An alternative technique of determining where to cut, by treatment of the strip with very dilute ninhydrin, described below for two-dimensional chromatograms, may be preferable when the spots are very close together. Variation in position of the spots caused by irregularities in the paper can then be followed.

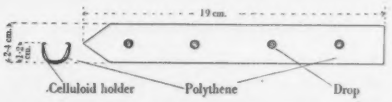

Fig. 4. Plan of polythene strip and cross-section of celluloid holder containing strip.

By attaching and squeezing a piece of cycle valve rubber the solution is forced out of the capillary on to a strip of polythene supported in a celluloid holder of semi-circular cross-section (Fig. 4). Polythene has been employed because its relatively unwettable surface prevents undue spreading of the drops and its flexibility makes for convenient transfer of the drops on to paper by 'printing'. After drying *in*

vacuo, the solid is dissolved in 20 μl. 6N-HCl by rubbing with a fine glass rod. The drop is then sucked, by means of the rubber tube, into another clean capillary whose ends are then sealed in the flame. Hydrolysis is effected by keeping the tube overnight at 105°. The hydrolysate is transferred back on to the polythene, dried *in vacuo* over KOH and taken up in 5 μl. of water. This drop, or a suitably spaced row of drops, if several analyses are being run in parallel, is now ready for transfer to the strip of filter-paper on which the final analysis is to be carried out. This is done by removing the polythene from its holder, inverting it and pressing it down on to the strip of paper ('printing') which lies on a clean sheet of glass.

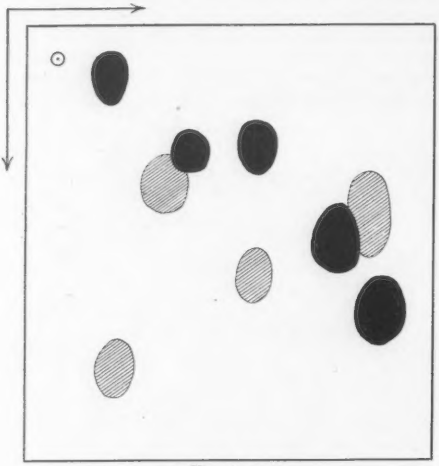

Fig. 5*a*.

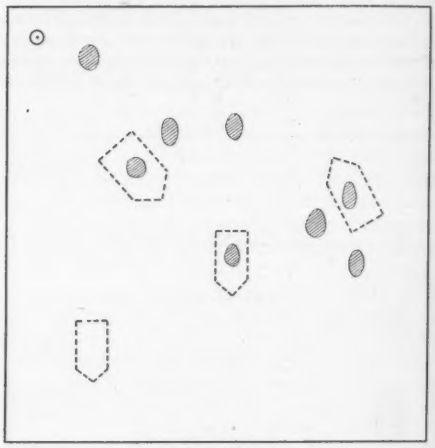

Fig. 5*b*.

Fig. 5. Diagram showing method of cutting of a two-dimensional chromatogram. (*a*) and (*b*) represent duplicate sheets, the solution to be analyzed being applied at the open circles. The arrows indicate the direction of development of the chromatograms in the two solvents. In the guide chromatogram (*a*), amino-acids are shown as black spots and peptides as shaded spots. After treatment with dilute ninhydrin, the duplicate sheet (*b*) shows faint spots (some may be barely visible) and is cut as shown by the dotted lines.

Where necessary, this final analysis can be done in two or more different solvents. Where this is intended 10 μl. or more of water are added to the dry hydrolysate and the solution is distributed between two or more filter-paper strips by means of a micropipette.

Two-dimensional chromatography

Duplicate two-dimensional chromatograms are developed at the same time, in the same chamber, from equal amounts of material. After thorough drying, one sheet is coloured with ninhydrin (0·1% in *n*-butanol). Only the stronger spots thus revealed would have contained enough material for successful analysis. The amount of material obtainable from one sheet is usually not enough for deamination as well as hydrolysis. The second sheet is sprayed with aqueous *n*-butanol containing not more than 0·01% of ninhydrin, dried and heated for 3 min. at 100°. The positions of the stronger amino-acid and peptide spots should be just visible. The areas required for analysis are cut out (Fig. 5*a* and *b*) and washed off as above. The small proportion of each peptide that has reacted with the ninhydrin does not interfere with the analysis. Previously, the positions of the stronger amino-acid spots were found by cutting pieces from one chromatogram using the coloured one as a guide and testing these with ninhydrin. Once the positions of the amino-acid spots had been found, the positions of the peptide spots could be inferred with fair accuracy. This technique has, however, been abandoned, firstly, because it consumed too much time and secondly, because the greater accuracy of the newer method permits smaller cuts to be made, a matter of particular importance where spots are rather near together.

Deamination and hydrolysis

The deamination of peptides is carried out on the polythene strip mentioned above, in 6N-HCl for 10 min. at 30–35°. After drying, each sample is dissolved in about 30 μl. of 6N-HCl (more, of course, if samples for simple hydrolysis are to be taken). The polythene strip, in its celluloid holder, with a number of samples to be deaminated, is pushed into a tube, warmed by a water jacket, through which are led the fumes produced by dropping concentrated HCl solution on solid $NaNO_2$. After 10 min., during which the oxides of nitrogen and NOCl dissolve in the drop (which turns brown at first and evolves gas whether amino-nitrogen is present or not), the strip is removed and dried *in vacuo* over KOH. After drying, more HCl is added and dried again to complete the removal of oxides of nitrogen. Finally, it is taken up in HCl and hydrolyzed and analyzed as described above.

These conditions give complete destruction of the amino-acid possessing the free amino group of peptides, but to ensure complete destruction of certain of the more

resistant free amino-acids, deamination must be continued for 30 min. Unfortunately, with peptides, some destruction of the amino-acids possessing a free amino group, occurs, perhaps as a result of premature hydrolysis and 30 min. treatment should only be employed where free amino-acids are known to be present in the fraction. It is always advisable to take at least twice as much material as for a simple hydrolysis.

Where several investigations, e.g. analyses in different solvents both of simple hydrolysates and hydrolysates after deamination, are to be run on a given fraction, it is preferable to use samples from the pooled fractions of several chromatograms rather than to perform each analysis on a fraction from a separate chromatogram. This avoids the danger that errors of cutting will give fictitious results in certain cases.

Effect of nitrous fumes and hydrolysis on some amino-acids and peptides. Though a fair proportion of pure tryptophan appears to survive hydrolysis, tryptophan which has been washed from a paper strip, no doubt because of the presence of degraded cellulose, is totally destroyed during hydrolysis. Even when the amino group is protected, as in a peptide, tyrosine and tryptophan are destroyed under the conditions of deamination described above. Synthetic tyrosylglycine gave the expected glycine spot, but glycyltyrosine showed a number of yellow and grey spots only. Leucyltryptophan and glycyltryptophan gave after deamination and hydrolysis only a very weak, slow-moving, brownish yellow spot.

When proline and proline peptides containing a free imino group, such as prolylvaline (Synge, to be published), are treated with nitrous fumes and then hydrolyzed, the yellow proline spot appears on the chromatogram of the products. Thus, prolylvaline gave proline and valine. This is presumably due to the formation of the *N*-nitroso derivative of proline during treatment with nitrous fumes, subsequent hydrolysis regenerating the imino group. Arginine and ornithine and peptides with the α-ornithine amino group either free or protected, e.g. ornithylleucine and α-valylornithine (Synge, to be published), gave after deamination and hydrolysis, faint proline spots and a number of faint unidentified spots on the chromatogram, in addition to the amino-acid spots expected from the peptides. The small amounts of proline were presumably formed by ring closure of the ornithine during treatment with nitrous fumes and subsequent hydrolysis. Free lysine gave two medium, unidentified purple spots.

Miscellaneous technical points

The fractions washed off the pieces of paper, as described above, usually contain after hydrolysis only two or three different amino-acids. Thus, identification can almost always be secured by using paper strips in two different solvents without recourse to the more elaborate two-dimensional technique. In the absence of aspartic and glutamic acids, *s*-collidine is a very valuable solvent. If it is run in an alkaline atmosphere, produced by a solution of 0·1% diethylamine dissolved in the water (2 l.) at the bottom of the box, the R_F of phenylalanine is increased so that on strips it almost coincides with tyrosine. On a collidine-diethylamine/phenol-gas-NH_3 two-dimensional chromatogram, phenylalanine does not overlap with any of the other amino-acids. 3% NH_3 is equally effective, but owing to its volatility this concentration is inconvenient to maintain and offensive in a box which has to be occasionally opened. Owing to its low volatility, a single addition of diethylamine will remain effective for several weeks. In the presence of diethylamine, chromatograms developed in *s*-collidine show 'fronts', particularly with the faster moving components. These fronts can be eliminated by a trace of HCN in the atmosphere. It has been found that the effect of one addition of HCN lasts for several weeks.

Small amounts of free acids when run in collidine-diethylamine pick up diethylamine and on treatment with ninhydrin appear as faint purple spots. H_2SO_4, $R_F=0{\cdot}45$; HCl, $R_F=0{\cdot}69$; acetic acid, $R_F=0{\cdot}60$. NaOH has been found to give a yellow spot $R_F=0{\cdot}24$ with or without diethylamine.

It has been found necessary to use benzyl alcohol only in glass boxes. When a rusty iron container was employed, serious elongation of the spots, which prevented full resolution, often occurred. The reason for this remains unexplained.

Where the amount of amino-acids available is too small for running on more than one strip, the separation of aspartic and glutamic acids in phenol can be achieved, after the separation of the fast running amino-acids in benzyl alcohol, by cutting the strip below the alanine position and developing backwards with the phenol. To do this, the spot to be analyzed is applied one third of the way down the strip. This technique is useful only in the absence of glycine, serine, threonine, alanine and cystine.

The use of Whatman no. 4 filter-paper, while in no way essential, markedly speeds up the development of the chromatograms (Consden *et al.* 1946*a*, *b*). Water-saturated *n*-butanol has been used for the ninhydrin solution because it gives rather stronger colours on the chromatograms. This may be because only in the presence of water can sufficient diffusion occur for all the amino-acids inside the cellulose fibres to react with the ninhydrin. The *s*-collidine should be pure and should remain colourless. Occasionally, a sample has been received from the manufacturers which is yellow and which deepens in colour on standing. Careful fractionation of such material fails to prevent subsequent colour development. To obtain satisfactory material, collidine (1 l.) is shaken with bromine (1–2 ml.), added in small quantities and allowed to stand overnight. It is then shaken with a few ml. of 40% (w/v) NaOH to remove HBr and distilled *in vacuo* over solid $Na_2S_2O_3$. The material so obtained remains colourless indefinitely.

SUMMARY

1. A method is presented for the identification of lower peptides present in protein partial hydrolysates, employing ionophoresis followed by partition chromatography on paper.

2. Peptide spots, obtained after chromatography on paper, are identified, after washing off the paper, by (*a*) hydrolysis, (*b*) deamination and hydrolysis. The amino-acids so formed are identified by chromatography on paper.

3. An apparatus, for the removal of salts from solutions of amino-acids and peptides, is described.

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Gramicidin S: the Sequence of the Amino-acid Residues

BY R. CONSDEN, A. H. GORDON, A. J. P. MARTIN, *The Wool Industries Research Association, Torridon, Headingley, Leeds* 6 AND R. L. M. SYNGE, *The Lister Institute of Preventive Medicine, London*

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We report here a study of the products of partial hydrolysis of gramicidin S by mineral acid acting at low temperature. Identification of these products has permitted an unequivocal formulation of the sequence of the amino-acid residues in this compound. A preliminary report of this work has been published (Consden, Gordon, Martin & Synge, 1946).

Gramicidin S seemed a particularly favourable object for study, since it had been shown to consist of the residues of the five amino-acids, *l*-valine (*V*), *l*-ornithine (*O*), *l*-proline (*P*), *l*-leucine (*L*) and *d*-phenylalanine (Φ) condensed together in equimolecular proportions; the presence of one free amino group/stoichiometric minimum unit, and the absence of free carboxyl groups, as well as the high optical rotation, were taken to indicate a closed peptide-chain (*cyclo*peptide) structure (Synge, 1945*b*). Subsequently Sanger (1946) established that the free amino group is the δ-amino group of the ornithine residue and the peptide chain therefore consists of amino-acid residues in α-linkage to one another. The crystallographic studies of Crowfoot & Schmidt (to be published) indicate that the molecule comprises either five or ten amino-acid residues; if the latter, the molecule also must possess a twofold axis of symmetry. This could occur only by twofold repetition of a particular sequence of the five different residues. It would be difficult, from studies of the later products of hydrolysis by acid, to distinguish between a given *cyclo*pentapeptide and the corresponding *cyclo*decapeptide structure, and we have not attempted to do so. The hydrolysis products which we have identified could all arise by the breakdown, without rearrangement, of the sequence of residues of the structure:

-α-(*l*-valyl)-*l*-ornithyl-*l*-leucyl-*d*-phenylalanyl-*l*-prolyl-
(-*VOL*Φ*P*-)

occurring either once or more often in a closed peptide chain. The fact that a high proportion of the possible hydrolysis products have been identified (some of them by more than one technique) gives a firm basis for assigning this structure to gramicidin S. That no products have been noted that are inconsistent with this structure, fits with the simple picture of the structure of gramicidin S which the crystallographic data suggest. A decision between the *cyclo*pentapeptide and *cyclo*decapeptide structures could perhaps be obtained by a study of the physical properties of gramicidin S in the dissolved state.

Knowledge of the structure of gramicidin S is likely to prove helpful for the more difficult problem of the nature of tyrocidine, which embodies the same peculiar amino-acid residues (Gordon, Martin & Synge, 1943*c*), and in which the δ-amino group of the ornithine is likewise free (Christensen, 1945). The present demonstration of the occurrence in nature of a *cyclo*peptide raises the question whether such structures may be embodied in proteins as sub-units (Chibnall, 1942, 1946). Such sub-units would have no characteristic terminal groups available for recognition, and would thus escape detection by methods employing this approach (Fox, 1945; Sanger, 1945).

The unequivocal results of the present study give increased confidence in the use of partial hydrolysis products (at least those formed by acid acting at low temperature) for the study of protein structure. The sort of control that is needed for such work was discussed by some of us six years ago (Gordon, Martin & Synge, 1941; cf. Synge, 1943); work with glutathione (reviewed by Fox, 1945) and gramicidin (Synge, 1944) and the results presented here provide further evidence for the assumption that no rearrangement of linkages occurs during acid hydrolysis. The rather

random character of the attack by acid on peptide structures leads to the conservation (as a variety of lower peptides) of all or most of the peptide bonds present in the original structure. This is an advantage in the study of molecules of relatively simple structure, such as gramicidin S. In the study of more complicated molecules the splitting of particular bonds and separation of the few resulting hydrolysis products seems more desirable as a first step. For this purpose proteolytic enzymes are promising agents, if it can be shown that they do not effect structural rearrangements by synthesizing new peptide bonds. Detailed studies of the action of enzymes on peptides of known structure should throw light on the question.

Techniques employed for study of the partial hydrolysates

Although we had an ample supply of gramicidin S, we deliberately developed and employed methods of analysis and identification applicable to smaller quantities of material, since in studies of other peptides and proteins only small quantities may be available. It is also to be expected that in partial hydrolysates the peptides incorporating particular bonds of the original structure will, as a result of lability under the conditions of hydrolysis, be present only in small quantity. Such peptides may, for structural elucidation, be of equal significance with those that are more abundant, and it is important to have methods suitable for their recognition. It should be noted that the significant experiments in the present work have been effected on altogether less than 10 mg. of gramicidin S. If the amino-acid residues present are known, and authentic specimens of peptides are available for comparison, the criteria of identity here employed seem equal or superior to the traditional criteria which require milligram quantities for their establishment. The identification of peptides by partition chromatography on paper is fully discussed in the preceding paper (Consden, Gordon & Martin, 1947). Four out of the five dipeptides expected to arise from partial hydrolysis were rigorously identified by these means, and the nature of two such tripeptides was also indicated.

In applying to simple peptides Sanger's (1945, 1946) methods of end-group assay (substitution of free basic groups with 2:4-dinitrophenyl (DNP-) radicals) it is possible, after the treatment with fluorodinitrobenzene, to extract the substituted peptides with organic solvents away from any material that has not reacted and which remains in the acidified aqueous reaction mixture. With hydrolysates of material that is known to be fully substituted, identification by paper chromatography of the free amino-acids could be expected to provide valuable information complementary to the identification of the DNP-amino-acids by chromatography on silica gel. Unfortunately, more exacting control experiments than those mentioned in our preliminary note (Consden, Gordon, Martin & Synge, 1946) have shown that traces of free amino-acids arise by breakdown of the DNP-amino-acids during hydrolysis by acid; the paper chromatograms with hydrolysates of DNP-peptides have therefore only proved of unequivocal value for the identification of δ-DNP-ornithine.

In work with DNP-amino-acids it was found that non-adsorptive silica gels, such as are useful for partition chromatography of acetylamino-acids (e.g. gels γ and ϵ, Gordon, Martin & Synge, 1944) do not permit useful separation of DNP-amino-acids employing the solvent-systems of Sanger (1945). When such gels are used in chromatograms, the DNP-amino-acids all run together as fast bands. Samples of silica gel provided by Dr Sanger gave, however, the expected separations. Their action must be attributed to the superposition of rather strong adsorption effects upon the effects due to distribution between the two liquid phases. With the structurally similar 2:4-dinitrophenylhydrazones of aliphatic carbonyl compounds, Roberts & Green (1946) have obtained useful separations with silica in simple adsorption chromatograms.

Other special techniques used in the present work, namely ionophoresis in silica jelly (Consden, Gordon & Martin, 1946) and quantitative determination of acetylamino-acids by partition chromatography with silica gel (Gordon, Martin & Synge, 1943*a*, 1944; Tristram, 1946) had already been employed with complete hydrolysates of gramicidin S (Synge, 1945*b*). In the present work the complete separation of free ornithine from peptides of ornithine by ionophoresis is a technical point worthy of note.

Identification of peptides present in the partial hydrolysates

From the results with two-dimensional partition chromatograms of unfractionated partial hydrolysates, the dipeptides α-(valyl)ornithine (*VO*), ornithylleucine (*OL*), leucylphenylalanine (*LΦ*) and phenylalanylproline (*ΦP*) were conclusively identified by the methods of Consden, Gordon & Martin (1947). The experiments also provided evidence for the tripeptides *VOL* and *ΦPV*, of which the constituent residues and the residues with free amino groups were identified. This evidence was more than sufficient to establish the sequence of the residues as *-VOLΦP-*. Experiments of the same type with the ionophoretic fractions showed that the basic peptide fraction (B) contained, as expected, only the peptides containing ornithine, while the other peptides were found in the neutral fraction (A).

Independent corroboration of the structure was obtained by further study of the basic peptide fraction (B) from the ionophoresis. Quantitative determination of the amino-acids in a complete hydrolysate of this fraction gave the molecular proportions to be $O > V > L > P$; Φ was absent. Since each peptide in this fraction must contain an ornithine residue, arguments analogous to those suggested for acetylpeptides by Gordon, Martin & Synge (1943*b*) can be applied. In this way are

excluded all but eight of the possible sequences, viz.: *-OVLP(Φ)-*, *-POVL(Φ)-*, *-PLOV(Φ)-*, *-LOVP(Φ)-*, *-VOLP(Φ)-*, *-LVOP(Φ)-*, *-PVOL(Φ)-*, *-PLVO(Φ)-*.

In the hydrolysate of the DNP-peptides derived from this fraction, DNP-valine but no DNP-leucine was found. This establishes the occurrence of the sequence *-VOL-* and limits the possible structures to *-VOLP(Φ)-* and *-PVOL(Φ)-*. Unfortunately, the destruction of DNP-proline during the hydrolysis prevented distinction by this method between the two possibilities. It is inferred that the ornithine peptide fraction contained the peptides *VO*, *OL*, *VOL* and *PVO*. The isolation of δ-DNP-ornithine from this material after treatment with fluoro-2:4-dinitrobenzene confirms Sanger's (1946) conclusion as to the mode of linkage of the ornithine in gramicidin S.

Course of hydrolysis of gramicidin S by acid at 37°

In the absence of quantitative data as to the amounts of each peptide present at each stage of the hydrolysis, the detailed kinetics of the process cannot be analyzed. However, the following facts appear noteworthy. (1) In the low temperature hydrolysis of gramicidin S the rupture of peptide bonds does not show the initially rapid rate followed by marked slowing-down that is characteristic of ordinary proteins (e.g. Gordon, Martin & Synge, 1941) and gramicidin (Synge, 1945*a*). With these, it may be supposed that some bonds in the structure are markedly more labile than others, and undergo preferential attack. With gramicidin S this effect is absent; indeed it may be supposed that the original molecule is more resistant to hydrolysis than are the split products immediately derived from it. Such a difference could be attributed to steric effects present in the relatively rigid *cyclo*peptide structure which disappear when the ring is opened. The kinetics of the attack by pepsin on native proteins has similar qualities (Tiselius & Eriksson-Quensel, 1939). (2) The proportion of free amino-acids formed during the hydrolysis is at all times less than expected, were the peptide bonds split at random. This agrees with previous data that, in general, dipeptides are more resistant to acid hydrolysis than higher peptides embodying the same residues (cf. Synge (1945*a*)). Dipeptides are, in fact, the predominant peptides in the later stages of hydrolysis, and four out of the five expected dipeptides have been identified. (3) Nevertheless, the bond *P-V* in the structure appears to behave in a special way. The dipeptide, *PV*, was not found in the partial hydrolysates, despite a search for it, controlled with synthetic material. Control experiments showed that had the dipeptide *PV* been liberated in quantities similar to those of the other dipeptides, a proportion of it, sufficient for detection, would have survived hydrolysis for 60 days. However, the two tripeptides, *ΦPV* and *PVO*, which incorporate this bond, appeared to be present in significant amounts. It must be assumed that both these tripeptides and their precursors break down in such a way as not to liberate a significant proportion of *PV*. Similar kinetic anomalies must, it seems, be anticipated in future structural investigations. Sufficient data do not yet exist for their prediction or rational interpretation in physical terms.

EXPERIMENTAL

Material. For the present study specimen III of gramicidin S (hydrochloride) was used. This was prepared by crystallization from a crude 4 % (w/v) ethanolic solution as issued for clinical use in the U.S.S.R. (series 9–1, Control No. 1857, 17/8/44). The recrystallized specimen had physical, chemical and crystallographic properties (Crowfoot & Schmidt, to be published) providing adequate identification with preparations previously studied (specimens I and II, Synge, 1945*b*).

Low temperature hydrolysis of gramicidin S

Gramicidin S, unlike gramicidin, is soluble in 10N-HCl. However, unless otherwise stated, hydrolysates employed in this work were prepared, as in previous studies with gramicidin and related peptides (Synge, 1944, 1945*a*), by keeping a 1·2% (w/v) solution of gramicidin S (hydrochloride) in 1:1 (v/v) glacial acetic acid : 10N-HCl at 37°. All glacial acetic acid used was purified by repeated crystallization followed by distillation from $K_2Cr_2O_7$. In view of the absence of tryptophan, no special precautions were taken to exclude air; the solution was kept in the incubator in a glass-stoppered vessel and samples for analysis were withdrawn at intervals. The solution darkened very slowly to a pale brown colour.

Further hydrolysis of the samples was effectively prevented by evaporating the samples to dryness *in vacuo* below 40°, dissolving the residues in water (with the earlier samples the addition of a little acetic acid was required to effect solution) and storing at 0°.

Table 1. *Course of low-temperature acid hydrolysis of gramicidin S*

	(As % of total N of gramicidin S)	
Days of hydrolysis	Amino-N	α-N of free amino-acids
0	15·3	0
2	18·9	3·4
10	28·3	6·2
25	50·3	14·5
40	57·0	23·4
54	61·0	27·8
Theoretical for complete hydrolysis (Synge, 1945*b*)	83·3	83·3

Table 1 shows the course of hydrolysis as indicated by determinations of amino-N (Van Slyke—0·5 hr. reaction time) and free amino-acids (ninhydrin-CO_2 according to Van Slyke, Dillon, MacFadyen & Hamilton (1941)—with 50 mg. ninhydrin at volume 2 ml. and pH 2·5). In the absence of information as to the liberation of the imino groups of

Table 2. *Amino-acids formed after hydrolysis and after deamination and hydrolysis of spots from two-dimensional chromatograms*

(*H* signifies hydrolysis only. *D* signifies deamination followed by hydrolysis. The number of crosses indicate the depth of colour given by each amino-acid spot with ninhydrin. Very roughly, each additional cross indicates two to three times as much material. Cut 3 for deamination missed the position of the peptide on the paper. The proline found on deaminating cut 1 was, presumably, formed from ornithine (cf. Consden *et al.* 1947). Cuts 1*a* and 1*b* (see Fig. 1*b*) were investigated separately but gave the same result. Cuts 3*a* and *b* were combined. See text for nomenclature of peptides.)

Cuts	1*a* and *b*		2		3*a* and *b*		4		5		6	
Amino-acids found	*H*	*D*	*H*	*D*	*H*	*D*	*H*	*D*	*H*	*D*	*H*	*D*
Ornithine	×××××	×××	××	.	×	.	.	.	.	.	.	.
Proline	.	×	.	.	.	.	×××	××	××	×	×	×
Valine	×××××	×	.	.	××	.	.	.	××	×	×	××
Leucine	.	.	××	×	××	.	.	.	×	.	××	.
Phenylalanine	.	.	.	.	.	.	×××	.	××	.	××	××
Peptides presumably originally present:												
Main component	*VO*		*OL*		*VOL*		*ΦP*		*ΦPV*		*LΦ*	
Minor component	.		.		.		.		*LΦ*		*ΦPV*	

proline residues it is not possible to calculate the average size of the peptides present in the mixture. The quantitative data in Table 1 are, however, consistent with the evidence from identification studies (below) that the peptides of the 58-day hydrolysates were predominantly dipeptides with a small proportion of higher peptides.

Analysis of 58-day hydrolysate of gramicidin S by chromatography on paper. Two lots of 1 mg. each of the material obtained by hydrolyzing gramicidin S for 58 days, as above, were subjected to two-dimensional chromatography on paper in *s*-collidine-3% NH_3 for 37 hr. followed by benzyl alcohol-HCN for 25 hr. One sheet was coloured with 0·1% ninhydrin in *n*-butanol (Fig. 1*a*) and using this as a guide (Consden *et al.* 1947), the amino-acid spots on the other were cut out and also coloured. In the light of this information the peptide spots were cut out (Fig. 1*b*), washed off, hydrolyzed and analyzed on paper chromatograms using *s*-collidine-3% NH_3 (Consden *et al.* 1947). As may be seen from Fig. 1*a*, spots 5 and 6 overlapped and complete separation could not be expected. Using similar amounts of material, two further chromatograms were developed and treated in exactly the same way except that the peptides were deaminated before hydrolysis. The amino-acids thus formed and the peptides which they had presumably constituted are shown in Table 2.

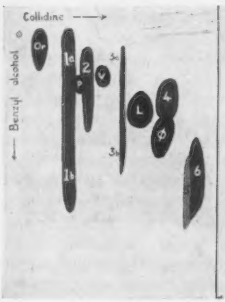

Fig. 1*a*.

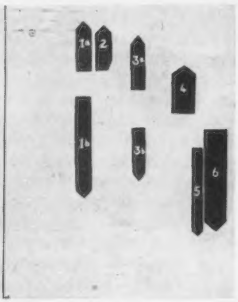

Fig. 1*b*.

Fig. 1. (*a*) Diagram of location of amino-acids and peptides on paper chromatogram of 58-day partial acid hydrolysate of gramicidin S. (*b*) Diagram of cuts made on duplicate chromatogram for isolation of the peptides. Numbers refer to peptide spots. Amino-acid spots are indicated by the following abbreviations: *Or*, ornithine; *P*, proline; *V*, valine; *L*, leucine; *Φ*, phenylalanine.

No attempt has been made to gauge the strengths of the peptides on the basis of the amounts of amino-acids set free on hydrolysis. Table 3 records the approximate relative intensities of the peptide spots as observed on the two-dimensional chromatograms serving as guides. Evidence from paper chromatograms for the occurrence of prolyl-valylornithine in a 5-day hydrolysate was obtained.

Table 3. *Relative intensities of dipeptide spots obtained from a 58-day hydrolysate of gramicidin S*

(The significance of the crosses is the same as in Table 2.)

Valylornithine	×××××
Ornithylleucine	×××
Leucylphenylalanine	×××
Phenylalanylproline	××

Comparison of the 58-day hydrolysate of gramicidin S with synthetic peptides by chromatography on paper. Two two-dimensional chromatograms were developed as described above, except that no NH_3 was used in the collidine direction. To one sheet, 1 mg. of the 58-day partial hydrolysate of gramicidin S was applied. To the second, was applied 0·5 mg. hydrolysate together with 100 μg. each of the synthetic peptides α-(*l*-valyl)-*l*-ornithine, *l*-ornithyl-*l*-leucine, *l*-leucyl-*d*-phenylalanine, *d*-phenylalanyl-*l*-proline and *l*-prolyl-*l*-valine (Synge, 1948). Excepting prolylvaline, which was observed as a yellow spot near to and to the right of the valine spot, no extra spots were observed on the sheet carrying the synthetic peptides on colouring with ninhydrin. The R_F values of the above peptides were determined on strips and are given in Table 4.

Table 4. *R_F values of dipeptides*

(The letters after the figures refer to colour given with ninhydrin; PG = pink grey; Y = yellow; without initials = purple.)

Solvent	*s*-Collidine	*s*-Collidine	Benzyl alcohol	*n*-Butanol
Addition	—	NH_3 (0·3%)	HCN	HCN
α-(Valyl)-ornithine	0·30	0·27	0·00	—
Ornithylleucine	0·38	0·39	0·01	—
Leucylphenylalanine	0·81	0·80	0·62	0·70
Phenylalanylproline	0·85 (PG)	0·83 (PG)	0·35 (PG)	0·56
Prolylvaline	0·60 (Y)	0·52 (Y)	0·11 (Y)	0·10 (Y)

Rate of hydrolysis of prolylvaline. Synthetic *l*-prolyl-*l*-valine (1·2 mg.) was dissolved in 1 ml. glacial acetic acid. 1 ml. 10N-HCl was added and the solution was kept at 37° and tested at intervals by partition chromatography on paper using collidine as solvent; 1·2 mg. ornithylleucine was treated similarly as a control. After 21 days *PV* and *OL* both gave strong spots, after 44 days *PV* was weak while *OL* was strong, and after 70 days *PV* was very faint while *OL* was still strong.

Ionophoretic fractionation of partial hydrolysate. Material resulting from a 58-day hydrolysis of gramicidin S as above was subjected to ionophoresis according to the procedure of Consden, Gordon & Martin (1946, method C). The analysis was carried out in a 4 cm. broad trough containing silica jelly impregnated with 0·1N-ammonium acetate and strengthened by incorporation of paper pulp. The jelly was covered with a glass lid before the beginning of the run. A quantity of material from the hydrolysate containing 6·9 mg. N was dissolved in 0·5 ml. water, and the resulting solution was neutralized (bromothymol blue) with strong ammonia solution (sp.gr. 0·88) and transferred quantitatively to the gutter in a total volume of 1·5 ml. The ionophoresis was allowed to proceed for 18 hr. with 2·3–2·6 V. cm.$^{-1}$. The electrodes were washed with 0·1N-ammonium acetate solution containing for the cathode, additional acetic acid (0·0125N) and, for the anode, additional ammonia (0·0125N). At the end of the run the lid was removed and a print from the jelly was taken on a filter-paper strip. The strip was dried, first in air, and then in a H_2SO_4-soda lime desiccator connected to a Hyvac pump for 1 hr. (to remove ammonium acetate, which is liable to give a background colour in the ninhydrin treatment). It was then sprayed with ninhydrin solution and heated. The resulting pattern is sketched in Fig. 1, together with the cuts decided on. It is of interest that, presumably owing to the presence of copper in the water-glass from which the jelly was made, the bands in cuts B and C in the jelly were coloured faintly blue. The jelly from each cut was dried in a vacuum desiccator, with high vacuum in the later stages to remove ammonium acetate, and the dry material was made into a column in a chromatogram tube (1 cm. internal diameter). H_2S was passed for a few minutes to render the copper insoluble, and then the material was extracted by passing seven columns' length of 1% (v/v) acetic acid. The extracts were evaporated to dryness *in vacuo* and the residues made up to volume in water and stored at 0° until required for study.

It was immediately established by paper chromatography (with *s*-collidine) that cut C contained free ornithine (apparently unaccompanied by other amino-acids and peptides) and that free ornithine was absent from cut B. It was inferred on theoretical grounds that cut B contained peptides of ornithine and cut A the 'neutral' amino-acids and peptides composed solely of them. The experiments described below show that this was indeed the case, and detailed confirmatory evidence was also obtained by paper chromatography.

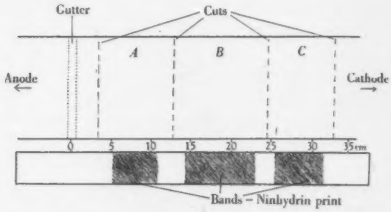

Fig. 2. Fractions resulting from ionophoresis of partial hydrolysate of gramicidin S.

Quantitative amino-acid analysis of the ornithine peptide fraction. One-fifth of the material from cut B of the ionophoretic fractionation described above was heated for 48 hr. at 110–115° in 3 ml. 6N-HCl in a sealed evacuated tube. The hydrolysate was acetylated and subjected to chromatographic analysis on silica gel exactly as in the quantitative determination of the amino-acid composition of gramicidin S itself (Synge, 1945*b*). Applying the same percentage corrections as were then used, the amino-acid composition of the fraction was found to be as given in Table 5. The absence of phenylalanine was further demonstrated by hydrolyzing with acid the material in the 'phenylalanine' and 'leucine' fractions and subjecting the hydrolysate to partition chromatography with benzyl alcohol on paper.

Table 5. *Amino-acid composition of the ornithine peptide fraction*

(Figures given are calculated for the whole fraction B resulting from the ionophoresis as % of the total N of the partial hydrolysate that was subjected to ionophoresis. No correction has been made for incomplete recovery from the ionophoretic fractionation; figures are therefore minimal.)

	Amino-acid N (as % of total N of gramicidin S)
Phenylalanine	0
Leucine	3·5
Valine	9·2
Proline	1·6
Ornithine	19·2

Investigation of the N-2:4-dinitrophenyl-(DNP-) derivatives of the ornithine-peptide fraction

DNP-amino-acids used in control experiments. DNP-*l*-proline, DNP-*dl*-leucine (Sanger, 1945), α-DNP-*dl*-ornithine, δ-DNP-*dl*-ornithine, δ-DNP-*l*-ornithine HCl.H_2O (Sanger, 1946) were made available by Dr F. Sanger. DNP-*dl*-valine was prepared from *dl*-valine in 90% yield by the procedure used by Sanger (1945) in making DNP-*dl*-alanine. The product had m.p. 182–184° (uncorr.; constant on recrystallization from methanol-H_2O). Abderhalden & Blumberg (1910) record 185°.

αδ-Di(DNP)-l-ornithine. 0·1 g. *l*-ornithine HCl was kept for 24 hr. with 0·4 g. $NaHCO_3$, 5 ml. water, 10 ml. ethanol, and 0·28 ml. fluorodinitrobenzene. The mixture was then evaporated *in vacuo* at room temperature. An orange gum separated, which later solidified. This was presumed to be the Na salt of di(DNP)-ornithine. The solid was triturated thoroughly with ether, to remove fluorodinitrobenzene, and the residue was then suspended in aqueous acetone and treated with excess 10N-HCl. Pale yellow crystals precipitated. Acetone was removed by partial evaporation *in vacuo* and after keeping overnight at 0° the crystals were filtered off, washed with water and dried in a vacuum desiccator. Yield was 0·29 g. Recrystallization was effected from hot acetic acid. The yield of crystals which formed on cooling was increased by diluting the mixture with water, in which the crystals were very insoluble. The material after drying in a desiccator had m.p. 155–157° (uncorr.; constant on recrystallization) and lost weight on further drying at 100° over P_2O_5 *in vacuo*, indicating the presence of water of crystallization. The material dried at 100° *in vacuo* appeared from the analytical data (Weiler, Oxford) to contain still at least one molecule of water of crystallization. (Found: C, 42·6; H, 3·42; N (Dumas), 17·2. $C_{17}H_{16}O_{10}N_6.H_2O$ requires C, 42·3; H, 3·74; N, 17·4%.)

Procedure for identification of DNP-amino-acids in acid hydrolysates of DNP-peptides. It was required to demonstrate the presence or absence of αδ-di(DNP)-ornithine, δ-DNP-ornithine, DNP-valine, DNP-leucine and DNP-proline. Preliminary experiments with the pure substances and with mixtures of them showed that the following procedure was satisfactory. For reasons already given, the silica gel used in this work was a preparation by Dr Sanger 'batch 7'. Each silica gel partition chromatogram employed 3 g. of this material made up with the appropriate solvent-system in a tube of 1 cm. internal diameter. The fractionation was based on the data of Sanger (1945), and followed his general procedure.

The mixture for study in aqueous 3·5N-HCl was extracted thrice with equal volumes of ether. The combined ether extracts contained all the DNP-amino-acids other than δ-DNP-ornithine which remained in the aqueous layer and was reserved for paper chromatography (see below). The combined ether extracts were evaporated to dryness *in vacuo*, and chromatographed on an ether-H_2O column. All the yellow material, which moved as a single fast band, was collected in the effluent from the chromatogram and evaporated to dryness *in vacuo*. It was then chromatographed on a chloroform-H_2O column (solvent, B.P. chloroform freed from ethanol by extracting it four times with equal volumes of water). The DNP-derivatives of leucine, valine and proline ran fast, and were collected as before; αδ-di(DNP)-ornithine moved as a yellow band having R_F 0·1–0·2. After the other DNP-amino-acids had left the column the di(DNP)-ornithine could be accelerated ($R=1$) by developing with 1% *n*-butanol-chloroform. The fast moving fraction from the chloroform column was then analyzed on an ethanol-ligroin-water column (Sanger, 1945). Three separate bands were obtained; DNP-leucine ($R=1$), DNP-valine ($R=0·4$–$0·6$) and DNP-proline ($R=0·2$–$0·3$).

The aqueous residue from the hydrolysate, after ether extraction, was evaporated to dryness *in vacuo*, the residue was dissolved in water and a suitable sample was applied to a paper strip and neutralized with NH_3-vapour; the strip was then developed as a chromatogram with *n*-butanol-H_2O according to the procedure of Consden, Gordon & Martin (1944). Under these conditions δ-DNP-ornithine ran as a yellow band (ahead of leucine, $R_F=0·6$ approx.). Unlike α-DNP-ornithine, the yellow band of δ-DNP-ornithine became purple on treatment with ninhydrin, and was thus readily recognized (cf. Sanger, 1946 for further data on the evolution of CO_2 by these compounds in the reaction with ninhydrin).

Control experiments. The following mixtures were heated in 4 ml. 5N-HCl for 48 hr. at 110–115° in sealed evacuated tubes: (X) 1·7 mg. αδ-di(DNP)-*l*-ornithine hydrate, 0·1 mg. *l*-proline, 0·33 mg. *dl*-leucine and 0·45 mg. *dl*-valine; (Y) 0·96 mg. δ-DNP-*dl*-ornithine, 0·78 mg. DNP-*dl*-valine, 0·41 mg. DNP-*dl*-leucine, 0·5 mg. DNP-*l*-proline, 0·1 mg. *dl*-leucine and 0·1 mg. *dl*-valine. The resulting mixtures were subjected to analysis as above. Much of the di(DNP)-*l*-ornithine in (X) had remained undissolved, and was filtered off before conducting the analysis. (X) yielded no coloured material in the aqueous residue from ether extraction, and revealed only one yellow band on the chloroform chromatogram, agreeing in rate with αδ-di(DNP)-ornithine. (Y) yielded a strongly yellow aqueous residue and paper chromatography showed a single yellow band, agreeing with δ-DNP-ornithine both in rate and in colouring with ninhydrin. Chromatography with silica gel revealed, on the ethanol-ligroin-H_2O column, only the bands of DNP-leucine and DNP-valine. These experiments indicate that, with the vigorous hydrolysis employed, DNP-proline is largely destroyed. αδ-Di(DNP)-ornithine, δ-(DNP)-ornithine, DNP-valine and DNP-leucine survive hydrolysis well, and no mono-(DNP)-ornithines are formed from αδ-di(DNP)-ornithine.

Preparation and study of DNP-derivatives of peptides in ionophoretic fraction B (ornithine peptides)

One-tenth of the extract from cut B of the ionophoresis experiment (see above) was allowed to react at room temperature (intermittent shaking) in 1·5 ml. 70% (v/v) aqueous ethanol with 0·04 g. $NaHCO_3$ and 0·03 ml. fluorodinitrobenzene. The mixture slowly turned yellow. After 48 hr. the mixture was evaporated *in vacuo* at room temperature, and the resulting aqueous emulsion, diluted to 7 ml. with water, was extracted thrice with equal volumes of ether to remove unchanged fluorodinitrobenzene. Very little yellow colour passed into the ether extracts, which were discarded. Residual ether was removed from the aqueous layer by evaporation *in vacuo* at room temperature. On acidification with 1 ml. 0·85N-HCl a partly crystalline yellow oil separated. On extracting the mixture with ether, most of the yellow colour passed into the extract, and after three such extractions the aqueous residue was colourless.

On evaporation of the combined ether extracts *in vacuo* the residue obtained was a yellow oil, containing some pale yellow dendritic needles, interspersed with more orange spherulites. The whole was readily soluble in ether, and all the yellow material was recovered as a fast-moving band from an ether-H_2O chromatogram. Attempts at analyzing the mixture on a chloroform-H_2O column were unsuccessful, since the yellow material, apart from a very small amount with $R=0{\cdot}6$, moved as a band whose front had $R=0{\cdot}1$, but which tailed excessively without resolving. All the yellow material was accordingly washed from the chromatogram with 1% *n*-butanol-chloroform. The extract was evaporated to dryness *in vacuo* at 60° and the residue was hydrolyzed with 4 ml. 5N-HCl in a sealed evacuated tube at 110–115°. The material dissolved only slowly; heating was continued for 48 hr. after solution was complete (16 hr.). At the end of this period there was only a very slight separation of crystals of αδ-di(DNP)-ornithine on cooling, and these dissolved during the subsequent extraction with ether. The analysis was continued as described above. Both the ethereal and aqueous solutions resulting from the initial extraction were strongly yellow. Silica gel chromatography indicated the presence of αδ-di(DNP)-ornithine and of DNP-valine, but DNP-leucine and DNP-proline were absent. δ-DNP-ornithine was identified by paper chromatography as the yellow constituent of the aqueous layer.

SUMMARY

1. Gramicidin S has been shown to yield, on hydrolysis with acid at 37°, the dipeptides α-(valyl)-ornithine, ornithylleucine, leucylphenylalanine and phenylalanylproline. The tripeptides α-(prolylvalyl)-ornithine, α-(valyl)-ornithylleucine and phenylalanylprolylvaline are also probably present. A variety of techniques employed for the identifications has yielded concordant results.

2. It is inferred that these peptides arise by the breakdown, without rearrangement, of the structure (-α-(*l*-valyl)-*l*-ornithyl-*l*-leucyl-*d*-phenylalanyl-*l*-prolyl-), occurring once or twice in a closed peptide chain. Gramicidin S, on the basis of the physical information so far available, is best formulated as the *cyclo*decapeptide or *cyclo*pentapeptide possessing this structure.

3. The fact that hydrolysis by acid at low temperature produces many peptides, all derived without rearrangement by the breakdown of a single structure, enables the identification of peptides in such hydrolysates to be used with increased confidence for studies of the sequence of amino-acid residues in peptide structures generally.

4. Some peculiarities in the kinetics of hydrolysis of gramicidin S are noted.

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Studies on the Polysaccharides of Capsulated Yeasts

By J. MAGER, *Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem*

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The polysaccharides of the cell wall of brewers' yeasts were investigated by Salkowski (1894) long before Avery, Goebel, Heidelberger and others began their well-known studies on the soluble substances of pneumococci. But while the study of bacterial polysaccharides was subsequently greatly expanded, little progress was made in research on yeast polysaccharides until Zechmeister & Tóth (1934) published a paper on the chemical structure of a polyose of the yeast cell wall (yeast 'cellulose'), later studied by Hassid, Joslyn & McCready (1941). Haworth, Hirst & Isherwood (1937) described the structure of a second yeast polysaccharide built up from units of mannose. Preliminary data on the carbohydrates of yeast species other than *Saccharomyces* were published by Kesten, Cook, Mott & Jobling (1930), and Tomcsik (1930) reported on the serological properties of 'gums' derived from a number of yeast species.

As far as we know no data have been published on the polysaccharides of capsulated yeasts. These are distinguished by the formation around the cell wall of voluminous transparent envelopes which remain unstained with ordinary staining techniques, but can easily be demonstrated in Indian ink preparations. This group of yeasts is classified with the genus *Torulopsis* (Lodder, 1938) and includes the pathogenic *T. neoformans* (syn. *Torula histolytica*, *Cryptococcus hominis*, etc.).

In a preliminary note (Aschner, Mager & Leibowitz, 1945), the phenomenon of the elaboration by capsulated yeasts of extracellular starch associated with a pentose-containing fraction was briefly recorded. Biological aspects of this phenomenon are discussed in another paper (Mager & Aschner, 1947); details of the chemical nature of these substances are considered in the present report.

EXPERIMENTAL

Isolation of the yeast starch

Material staining blue with iodine was isolated from a saprophytic yeast identified as *Torulopsis rotundata*. For the preparation of this material cultures were grown under continuous aeration in a medium of the following composition: $(NH_4)_2SO_4$, 0·1%; glucose, 1%; KH_2PO_4, 0·1%; $MgSO_4$, 0·05%; aneurin, 20 μg./100 ml. After incubation for 4–5 days at 30° the cultures were killed by heating at 60–80° for 0·5 hr. and the cells removed by centrifugation. The supernatant liquid was concentrated over a steam bath to about one fourth of its original volume and, after cooling, trichloroacetic acid was added to a final concentration of 4 g./100 ml. The slight precipitate which usually appeared after standing overnight in the refrigerator was centrifuged off and discarded, and 3 vol. of ethanol were added to the supernatant. A copious threadlike precipitate was obtained, which was repeatedly washed with 60–70% ethanol acidified by a few drops of acetic acid. When washing fluid was free from reducing sugar, the material was washed three times with absolute ethanol, then with warm ether, and was finally dried in a desiccator. About 1·5 g. of material (1·5% of the original sugar content of the medium) was recovered from a volume of 10 l. of medium.

General properties of the substance

The isolated material showed the characteristic properties of a polysaccharide, viz. it did not pass through cellophan membranes, gave a positive Molisch reaction and failed to reduce alkaline copper solutions except after hydrolysis with dilute acid. The solubility of the substance in water was very slight, but highly viscous and opaque solutions could be obtained by heating for 0·5 hr. under water in an autoclave at 120° at pH 8. The dissolved material was retained by bacterial filters (Berkefeld N and Seitz E.K.). It could be precipitated from aqueous solution by ethanol, acetone or Fehling's solution. The material was free from protein and amino-acids, as determined by the ninhydrin and biuret reactions, and from amino hexoses as determined in its hydrolysate by the reagent of Elson & Morgan (1933). Samples purified by dialysis were N free when tested by the Lassaigne and the micro-Kjeldahl tests, and were ash free on combustion. The blue colour formed with a solution of iodine in potassium iodide disappeared on heating and reappeared on cooling. The quality of the colour was similar to that of the amylose-iodine complex and free from the violet tint characteristic of unfractionated starch containing amylopectin. The light extinction of the iodine complex, measured in a Pulfrich Stufenphotometer, showed a maximum with filter of 610–630 mμ. wave-length in agreement with the value of extinction for amylose-iodine as reported by Kerr & Trubell (1943) on the basis of spectrophotometric determination. According to the same authors the iodine complex of amylopectin presents a maximum extinction at 700 mμ.

Estimation of reducing sugar content. Ash-free material (51·6 mg.) was hydrolyzed in 10 ml. of N-HCl for 3 hr. After neutralization with Na_2CO_3, the solution was made up with water to 20 ml. and the reducing sugar content determined by the method of Somogyi (1945). One ml. of solution containing 2·58 mg. of test material had a reducing value equivalent to 1·69 mg. glucose, i.e. 65·5 % of the weight of the substance. The same result was obtained when HCl was replaced by H_2SO_4 or when the strength of the acid was raised (5 N). Essentially the same result was obtained when the heating time was prolonged to 5 hr.

Chemical nature of the sugar component

Fifteen ml. of a solution of the hydrolyzed material, containing 0·32 mg. of reducing sugar (calc. as glucose)/ml., were fermented with washed commercial bakers' yeast. At the same time a control experiment was done with pure glucose. After 0·5 hr. the solutions were freed from cells by centrifugation and clarified by $Zn(OH)_2$ according to the method of Somogyi (1945). The reducing sugar content was determined, with the following results: control test, 0; test of the unknown, 0·17 mg. Thus, 46·3 % of the reducing sugar in the preparation was fermented by bakers' yeast.

To ascertain whether the residual reduction was due to galactose, further fermentation tests were carried out using strains from laboratory cultures of *Torulopsis utilis*, which ferments glucose, fructose and mannose but not galactose; and a strain of *Torulopsis* sp., which ferments galactose as well as the above-mentioned monosaccharides. In a control experiment a mixture containing hydrolyzed amylose, prepared by the method of Ling & Nanji (1923), and galactose was used. The results showed that the galactose-fermenting and the galactose-non-fermenting yeasts each removed about the same amount of sugar. In one experiment the fermentable sugar fraction constituted about 45 % of the total reducing sugar. However, the proportion of the fermentable fraction in different preparations varied from 25 to 50 % of the reducing value. It may, therefore, be concluded that the blue-staining carbohydrate consists of at least two fractions which can appear in varying proportions in different preparations. One fraction, composed of fermentable sugar, will be designated '*A*' and a second, composed of unfermentable sugar, will be designated '*B*'.

Action of amylases on the blue-staining material

Suitably buffered 0·05 % solutions of the yeast carbohydrate were treated with the following amylase preparations: salivary amylase prepared by the method of Hanes & Cattle (1938); aqueous extract of ground malt, and commercial preparations of taka-diastase and bacterial amylase manufactured by Wallerstein Laboratories. All these amylases caused the blue staining of the material with iodine to disappear. The achromic point was reached much more slowly with yeast polysaccharide than with soluble or rice starch.

In a quantitative experiment, 10 ml. of a strong solution of yeast starch, containing the equivalent of 5·25 mg. reducing sugar/ml. (calc. as glucose), was treated with 3 mg. of a salivary amylase preparation in the presence of 0·01 N-acetate buffer pH 6·1 and 3 mg. of NaCl. The mixture was incubated at 37° with constant agitation. After 6 hr. the amount of reducing sugar set free by the action of amylase was found to be 1·04 mg./ml., i.e. 19·8 % of the total reducing value. The actual amount of starch present in the material could not, however, be evaluated since the amount of amylase-resistant dextrin was not known.

Decomposition of yeast starch by β-amylase. β-Amylase free from reducing sugar was prepared from wheat by the method of Hanes & Cattle (1938). Yeast carbohydrate (115 mg. containing 69 % reducing sugar and 26·3 % fermentable sugar) was dissolved in 45 ml. of water by autoclaving at 120° for 30 min. To the viscous solution were added 3 ml. of 0·1 N-acetate buffer pH 4·8, and 5 mg. of β-amylase. After incubation for 24 hr. at 30° the solution still gave a blue colour with iodine. Reducing sugar was determined in 5 ml. portions before and after fermentation for 2 hr. with bakers' yeast. The results showed 1·46 mg. reducing sugar (as maltose) before, and 0·12 mg. after, fermentation. It may be assumed that the non-fermentable reducing product of β-amylase hydrolysis is dextrin. Deducting this value from the total of reducing sugar released by β-amylase gives 1·34 mg. maltose equivalent to 1·41 mg. glucose. The amount of fermentable sugar in this preparation equalled 26·3 % of the weight of the substance. Hence, on the assumption that the fermentable sugar is wholly derived from the starch fraction, complete hydrolysis by β-amylase should have set free from 11·5 mg. of the substance an amount of maltose equivalent to 3·02 mg. glucose. The amount actually obtained (1·41 mg.) represents, therefore, 46·7 % of the starch fraction. (Since it was subsequently found that the capsular material which appears in association with the yeast starch contains about 15 % of zymohexose, the real value for maltose split off by β-amylase would be somewhat higher.)

It has recently been claimed (Wilson, Schoch & Hudson, 1943) that the presence of fatty materials in corn starch prevents the complete conversion of its amylose component by β-amylase or *B. macerans* amylase. After the removal of lipids with 80 % (v/v) methanol, complete enzymic hydrolysis of corn starch-amylose can be obtained. We, therefore, repeated the experiments described above using a preparation of yeast starch which had previously been treated with 80 % (v/v) methanol. The results were in no way altered.

Examination of the product of amylase action. It remained to be determined whether or not the fission product obtained by amylase action was maltose. The colorimetric method described by Hestrin & Mager (1946, 1947) for the identification of sugars was used for this purpose.

Absolute ethanol (3 vol.) was added to a solution of yeast starch treated with salivary amylase as described above. The resulting precipitate was separated by centrifugation and the supernatant fluid evaporated to dryness. The residuum was redissolved in 5 ml. of distilled water. This solution was found to contain 4·3 mg./ml. of reducing sugar calculated as maltose. The solution (0·6 ml.) was transferred to a Thunberg tube and evaporated to dryness. To the dry residue were added 4 ml. of phosphoric acid (85%), and the solution heated in a boiling water bath for 30 min. No colour formation occurred. Finally, about 10 mg. of pyrocatechol was added to the phosphoric acid solution and the heating continued for 10 min. A red-violet colour then appeared and proved to be identical in absorption curve with that of glucose and glucose anhydrides.

Since the product of amylase hydrolysis gave a negative reaction with the monose reagent of Tauber & Kleiner (1932), it was obvious that the sugar in question must be an oligosaccharide. Direct support for its identification as maltose was obtained by the preparation of a phenylosazone, which was soluble in hot water and yielded yellow crystals of the microscopic form described by Hassid & McCready (1942) for maltosazone.

Search for zymohexoses other than glucose

The experiments so far reported do not exclude the possibility that the '*A*' fraction contains fructose or mannose in addition to glucose. Quantitative tests for fructose (Seliwanoff, Pinoff) were negative. Aldose determination of the hydrolyzed material by the iodometric method of MacLeod & Robison (1929) gave a result closely approximating the value for total reducing sugar obtained by Somogyi's method.

As a test for mannose, the isolation of mannose phenylhydrazone, a substance distinguished by its insolubility and the rapidity of its formation in the cold, was attempted. Although about 200 mg. of hydrolyzed material were used for this test, no precipitate other than a small amount of a non-crystalline red sediment was produced with phenylhydrazine. The result of this test was, therefore, read as negative.

Identification of fraction 'B'

The hydrolyzed material freed from zymohexose (fraction '*B*') was subjected to various qualitative tests. As little as 1–2 mg. of material gave a strong positive reaction with Bial's orcinol reagent, which gives positive tests with uronic acids and methylpentoses as well as with pentoses; but the presence of compounds of the first two groups was excluded by specific tests. The naphthoresorcinol test of Tollens was applied for uronic acid and the acetone test of Rosenthaler for methylpentose (cf. Van der Haar, 1920; Browne & Zerban, 1941). Both tests were negative even when 50–100 mg. of hydrolyzed material was employed. On the other hand, the aniline acetate test of Schiff, which is fairly specific for pentose, was strongly positive with 1–2 mg. of the hydrolyzed polysaccharide. In every case control tests on solutions of known sugars including xylose, glucuronic acid and rhamnose were run.

The results indicated clearly that the Bial positive sugar in fraction '*B*' is a pentose. The question arose whether all the unfermentable sugar fraction consists of pentose.

The material (21 mg.) was hydrolyzed with hot N-HCl for 2·5 hr. After neutralization the solution was adjusted to 50 ml. The reducing sugar content was then determined on 2 ml. of this solution. In another 2 ml. diluted 1 : 80 the pentose content was estimated by the method of Mejbaum (1939). The quantitative method of Mejbaum for pentose was chosen for its simplicity and because it affords results which are independent of the configuration of the pentose (Schlenk, 1943). The photometric readings were made in a Lumetron apparatus using filter 610 mμ.

The following results, calculated on the volume after hydrolysis and adjustment to 50 ml., were obtained: total reducing sugar: 0·215 mg./ml.; pentose: 0·206 mg./ml. The values for pentose and total sugar were thus practically identical, the slight difference observed being within the limits of experimental error. The conclusion seems warranted that all of the unfermentable sugar in fraction '*B*' is pentose.

Determination of the specific optical rotation of the hydrolyzed material

The optical rotation was determined in a 1 dm. tube at 18° after the addition of alkali to a concentration of 0·1N. The results are summarized in Table 1.

Table 1. *The specific rotation of the unfractionated hydrolysate of carobohydrate material and fraction '*B*'*

	Sugar conc. (*c*) (%)	Optical rotation (*α*)	$[\alpha]_D = 100\alpha/lc$
Unfractionated material	1·28	+0·60°	+46·8°
Fraction '*B*'	0·67	+0·13°	+19·4°

The rotation values of different dextrorotatory pentoses, as reported in the literature, are as follows: D(+)-xylose +10°; D(+)-lyxose +13·9°; L(+)-arabinose +105·5°; L(+)-ribose +23·7°. The rotation value of fraction '*B*' is, therefore, nearest to D-xylose and L-ribose. However, the presence of a certain amount of another pentose, in particular of

Table 2. *Fermentation of various pentoses and of the hydrolysate of fraction 'B' by bacterial species of the family* Enterobacteriaceae

Species of bacteria	L-Arabinose	D-Arabinose	D-Xylose	D-Ribose	D-Lyxose	Pentose of the capsule material
Salmonella choleraesuis	–	–	A, G	A, G	±	A, G
S. schottmülleri	A, G	–	A, G	A, G	±	A, G
S. typhimurium	A, G	–	A, G	A, G	–	A, G
S. paratyphi (A)	A, G	–	–	A	±	–
S. pullorum	A, G	–	–	A, G	–	–
Shigella ambigua	–	–	–	A	±	–
Shig. sonnei	A	–	–	–	±	–
Proteus Oxk	–	–	–	–	–	–
Escherichia coli	A, G	A, G	A, G	A, G	±	A, G
Eberthella typhosa str. Bhatnagar	–	–	A	A	A	A

Explanation of signs: A = acid; G = gas; ± = weak acidification; – = no acid nor gas.

lyxose, cannot be excluded. (Gulland & Barker (1943) found a small amount of L-lyxose in commercial preparations of yeast nucleic acid.) The higher rotation of the unfractionated material, on the other hand, is consistent with the presence of glucose in high concentration.

Microbiological identification of the pentose component

With only a limited amount of material at our disposal detailed investigation of the configuration of the pentose by the accepted preparative methods was not feasible. We attempted to avoid this difficulty by means of a microbiological method which could be applied to small quantities. Although many microbiological methods have been suggested by various workers for the identification of different sugars (cf. Harding & Nicholson, 1933) no complete scheme for the identification of pentose configurations has been elaborated.

It is known that various species in the family of Enterobacteriaceae differ from one another in their ability to ferment D-xylose and L-arabinose. However, information concerning the behaviour of bacteria of this group towards the other configurational isomers of pentose has not, to our knowledge, been published. For this reason we examined the behaviour of a number of species towards a collection of pentoses.

The tests were made in test tubes (small size) containing 3 ml. of semi-solid agar of the following composition: peptone, 0·2%; Na_2HPO_4, 0·07%; NaCl, 0·1%; Andrade indicator; and 0·1–0·2% of the sugar to be examined. It was found that the given low concentration of sugar sufficed for acid and gas formation in positive cases.

The ability of the bacteria to ferment the pentose contained in fraction 'B' of the carbohydrate material of *T. rotundata* was determined as follows: The hydrolyzed material was adjusted to pH 7·0 and freed from zymohexoses by yeast fermentation. The cleared solution was diluted to a sugar concentration of 0·4% (calculated as glucose). The solution was sterilized by passing through a bacterial filter and was added to an equal volume of double strength semi-solid agar.

The results of these examinations, summarized in Table 2, show that the fermentation behaviour of the pentose component constituting fraction 'B' was identical with that of D-xylose.

Identification of fraction 'B' with the capsular substance of Torulopsis rotundata

It was mentioned in a preliminary note (Aschner, Mager & Leibowitz, 1945) that under certain conditions a pentose-containing polysaccharide which does not stain blue with iodine can be isolated from cultures of *T. rotundata*. The production of the iodine-staining material is suppressed when a source of nitrogen is employed which does not allow acidification of the culture medium such as, for instance, 0·05% urea, 0·1% KNO_3 or 0·1% asparagine. The substance produced under these conditions could be shown to be derived from the capsule of the yeast, since it appeared in cultures of all the non-fermenting capsulated yeasts, but failed to appear in cultures of their capsule-free variants (Mager & Aschner, 1947).

Thus it occurred to us that fraction 'B' might be identical with the capsular substance. The experimental data summarized in Table 3 seem fully to confirm this assumption.

Table 3. *Composition and properties of the capsular substance*

(i)	Hot Fehling's solution: precipitate; no reaction
(ii)	Total reducing sugar of the hydrolysate: 58–67% of the weight of the substance
(iii)	Fermentable fraction of the total reducing sugar: 13·5–15%
(iv)	$[\alpha]_D$ of the unfermentable 'B' fraction: $+20·6 \pm 2·2°$
(v)	Action of amylase: not acted on
(vi)	Qualitative tests on the unfermentable fraction: pentose
(vii)	Mejbaum's pentose estimation: 61% of the weight of the substance
(viii)	Microbiological test for pentose: D-xylose
(ix)	Tests for mannose, fructose and galactose: negative

One additional fact came to light in these experiments, viz. that the capsular substance itself con-

tains a fraction composed of a zymohexose obviously identical with glucose. This fraction however must be different from starch, since it does not impart a blue colour to the material when treated with iodine, and since it is not attacked by amylases.

Elementary analysis of the capsular substance. In various preparations the amount of the reducing sugar yielded by hydrolysis constituted 60–70% (w/w) of the substance. This seems to indicate that the material isolated from *T. rotundata* contains an additional non-carbohydrate fraction. Since, however, it might be that the apparent deficit in reducing sugar was due to incomplete hydrolysis or to some other technical factor, the chemical nature of the material as a whole was examined by an elementary analysis.

Duplicate microanalyses carried out on a sample of capsular substance yielded the following results:

Material dried at 96°: C, 38·1, 38·5; H, 6·7, 6·7%; N, nil; ash, nil.

Material dried at 108°: C, 38·9, 38·8; H, 7·2, 7·4%.

The theoretical values for hexosan and pentosan are:

Hexosan, C, 44·4; H, 6·17%; *pentosan*, C, 45·45; H, 6·0%.

There is thus considerable divergence between the experimental findings and these theoretical values.

Isolation of the yeast starch free from capsular fraction 'B'

Several attempts to prepare a yeast starch preparation free from accompanying capsular substance were unsuccessful. As mentioned above, hot Fehling's solution precipitated the capsular substance. We therefore attempted to isolate starch from the supernatant fluid after treatment with Fehling's solution. It was found, however, that the bulk of the starch fraction was trapped by the precipitate of the capsular substance.

We succeeded in separating the yeast starch from the capsular material by a freezing-out procedure. Culture medium in which *T. rotundata* has been grown was freed from cells and concentrated to about one tenth of its original volume. After freezing for 48 hr. the starch constituent settled out whereas the capsular substance remained in solution. In this way about 80 mg. of pure yeast starch were recovered from 3 l. of culture medium. Solutions of the pure material were distinguished by rapid retrogradation on standing. In contrast to the mixed preparation the pure yeast starch was precipitated by an excess of iodine solution and not by Fehling's solution. On hydrolysis 22 mg. of the material yielded 20·5 mg. of reducing sugar (*c.* 90% of theory). The hydrolyzed sugar was completely fermented by bakers' yeast.

Examination of the carbohydrate materials produced by other species of capsulated yeasts

Since the ability to produce an extracellular material staining blue with iodine is shared by non-fermenting capsulated yeasts as a group (Mager & Aschner, 1947) the experiments described were repeated on a number of other capsulated yeasts. The results were not essentially different from the findings with *T. rotundata* reported above. The blue-staining material and the capsular substance elaborated by the pathogenic yeast *T. neoformans* (Palestine strain) was essentially identical with the analogous substances from *T. rotundata*. In examining the carbohydrate materials isolated from other capsulated non-fermenting species we confined ourselves to tests of their ability to stain blue with iodine, of their susceptibility to hydrolysis by salivary amylase and of their association with a material containing pentose. In all these respects the results with the following species of *Torulopsis*, comprising all those tested, were the same as with *T. rotundata*: *T. innocua*; *T. mucorugosa*; *T. liquefaciens*; *T. neoformans*, str. Sanfelice; *T. neoformans*, str. Tartar; *T. neoformans*, str. Freeman & Weidman.

Demonstration of a starch-synthesizing enzyme in cells of Torulopsis rotundata

As reported by Mager & Aschner (1947), resting cells of *T. rotundata* are incapable of producing starch. From this it was inferred that the elaboration of starch is in some way linked with vital anabolic processes of the proliferating cells. Nevertheless, it appeared probable that the enzymic apparatus of starch formation might be set free by destruction of the cellular organization. Attempts to release the enzyme were first made by autolyzing the cells, but the cells of *T. rotundata* proved to be extremely stable in this respect, since cells kept under toluene at 37° showed signs of cell disintegration only after a period of at least 2 weeks.

The following experiment was then undertaken: cells of *T. rotundata* grown at 30° for 3 days on peptone glucose agar, i.e. under conditions which do not permit the production of starch, were harvested with aseptic precautions and washed free of the culture medium. Glucose or glucose-1-phosphate (0·5%) was added to thick suspensions of these cells in 5 ml. acetate buffer solutions of graded pH, and the mixtures incubated under toluene. Samples (0·2 ml.) were withdrawn and tested for starch with iodine solution. After 20 days of incubation, tests showed that starch was produced with Cori ester as substrate over the range pH 4·5–5·9, but not with glucose. In both instances marked autolysis was evident microscopically. It seems obvious, therefore, that the disintegrating cells release a starch-producing enzyme which acts as a phosphorylase.

This enzyme could also be demonstrated in cell preparations of *T. rotundata* by another method. Cells grown on glucose peptone agar were dried from the frozen state in a Cryochem apparatus. About 0·5 g. of the dried material, washed twice with acetone and ether, was mixed with an equal amount of glass powder and ground for several minutes in a glass mortar with 5 ml. of water. After centrifugation the supernatant was added to an equal amount of 1% of either glucose or Cori-ester solution in acetate buffers of varying pH. After 5–10 min. the solutions containing Cori-ester at pH 4·5–5·9 turned blue when tested with iodine.

DISCUSSION

The chemical analysis of materials which stain blue when treated with iodine and which were isolated from liquid cultures of the yeast *T. rotundata* and related capsulated species, shows that these materials are mixtures of at least two different carbohydrate substances. One is a polyglucoside similar to plant starch, and the other is a substance derived from the yeast capsule. The physical and biochemical properties of the polyglucoside as examined in the presence of the capsular substance are not identical with the characteristics of either of the two fractions of plant starch. In its reaction to iodine, its low solubility and its tendency to retrogradation in neutral solutions, yeast starch resembles amylose (β-amylose, amyloamylose). On the other hand, the incompleteness of its conversion by β-amylase suggests that it should be classified with amylopectin (α-amylose, erythroamylose; Samec & Waldschmidt-Leitz, 1931; Hanes, 1937; Hassid & McCready, 1943). It seems likely that yeast starch is a substance intermediate between α-amylose and β-amylose, and possibly identical with the γ-amylose component of corn starch described by Kerr & Severson (1943). However, the possibility cannot be ruled out that the properties of yeast starch in the examined preparations were dependent on the close association of the starch fraction with capsular substance. It is conceivable that amylase cleavage is hindered by the presence of the capsular material.

Cori, Schmidt & Cori (1939) have shown that phosphorylase isolated from skeletal muscles produces pure amylose, whereas live muscle produces glycogen. To explain this difference Cori & Cori (1944) (cf. Cori, Swanson & Cori, 1945) put forward the theory that glycogen is produced by the simultaneous action of two enzyme systems, the first forming the straight chain of the amylose molecule by binding glucose units in 1:4 bonds, and the second attaching to this chain the branches characteristic of glycogen which are linked by 1:6 bonds. In the course of making enzyme preparations, the 'branching factor' may be lost with the result that only the 'straight chain factor' remains in the enzyme preparation finally obtained. A similar view was suggested by Haworth, Peat & Bourn (1944; cf. Bourn & Peat, 1945) to explain the absence of the amylopectin-forming factor in phosphorylase prepared from potatoes by the method of Hanes (1940).

Speculating along such lines we might assume that the 'branching factor' in the case of the yeast cells is destroyed or inactivated at the low pH at which starch production occurs, while the 'amylose-producing factor' retains its activity in the same medium. Since the cell wall is injured by the acid reaction of the medium, the amylose is able to pass out of the cell and accumulates in the capsule and in the medium. The assumption that the cell wall suffers damage under conditions of progressive acidification is supported by microscopic observations (Mager & Aschner, 1947).

The hypothesis considered above does not explain the fact that starch production is limited to a special group of yeasts. Another interpretation which offers a more satisfactory explanation for the whole phenomenon deserves consideration. According to this hypothesis it is assumed that starch is the precursor of the yeast capsule pentosan. Under normal growth conditions the starch may be transformed into pentosan by oxidation of the glucose units at carbon 6 to glucuronic acid, with subsequent decarboxylation of the glucuronic acid to D-xylose. This transformation may be supposed to be paralyzed at an acid pH, resulting in the fixation of the intermediary starch. It will be recalled that a mechanism entailing mild oxidation and decarboxylation has been envisaged by various authors to explain the mode of formation of the pentose constituent of hemicellulose and pectins in plants (cf. Norman, 1929). Such an interpretation would explain two facts which are not well accounted for in the alternative hypothesis: the selective accumulation of the starch in the capsule, and the restriction of the phenomenon to capsulated yeasts.

SUMMARY

1. The properties of a carbohydrate material which stains blue with iodine, isolated from cultures of *Torulopsis rotundata*, *T. neoformans* and other capsulated yeasts, are described.

2. The material is a mixture of starch and pentosan derived from the capsule of the yeast.

3. The capsular material consists of a large carbohydrate fraction (*c.* 60–70%) and a non-carbohydrate one. The carbohydrate constituent is composed of a zymohexose (15%) and a pentose (85%). The reported experiments have led to a tentative identification of the zymohexose as glucose and of the pentose as D-xylose.

4. The demonstration in cells of *T. rotundata* of a phosphorylase which synthesizes starch is recorded.

I wish to express my sincere thanks to Dr M. Aschner and Dr J. Leibowitz for their constant guidance during the progress of this work. I am indebted to Dr S. Hestrin for his helpful suggestions, to Dr J. Leibowitz for providing a specimen of amylose, and to Dr F. Bergmann for kindly performing the microanalyses.

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Determination of Pregnanediol in Urine for Diagnostic Purposes

By DORA HUBER, *Laboratory of the University Gynaecological and Obstetrical Clinic, Zurich, Switzerland*

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Pregnanediol, excreted in urine in the form of sodium pregnanediol glucuronidate, is the chief metabolite of the corpus luteum hormone, progesterone, and the relation between pregnanediol excretion and corpus luteum activity makes it important to have a reliable and convenient method for determination of pregnanediol. It is physiologically inactive, in spite of its close relation to the hormone, and biological tests cannot, therefore, be used. Pregnancy may be diagnosed at an early stage from the pregnanediol excretion, which is, during the first 2 months' gestation, about equal to the maximum in the latter half of the menstrual cycle, rises progressively to a maximum and then falls precipitously after delivery. Pregnanediol excretion drops to a low level when miscarriage is imminent (cf. Guterman, 1946). Also, the level of pregnanediol excretion may be considerably increased in adrenal cortical carcinoma or adrenal hyperplasia (Venning, Weil & Browne, 1939).

Various methods for the determination of pregnanediol in urine have been proposed, but they are either too complicated or too inaccurate for routine clinical use. The method of Venning & Browne (Venning, 1937, 1938; Venning & Browne, 1940) and the modification of Westphal (1944) do not, in the author's experience, allow the determination of less than 5 mg. of sodium pregnanediol glucuronidate,

because this substance is not quantitatively precipitated by acetone-water. The method of Allen & Viergiver (1941), depending on the titration of glucuronic acid liberated from the glucuronidate, yields high results, because of the precipitation of other glucuronidates at the same time. The method of Astwood & Jones (1941) is simple and reproducible, but only 70–80% of the pregnanediol present is recovered. Guterman (1944) modified this method and estimated the pregnanediol colorimetrically: the method was further developed by Morrow & Benua (1946) and by McCormack (1946).

The author's original intention was to use Guterman's method for the assay of pregnanediol. It is sufficiently rapid for routine determinations, but it was found impossible completely to precipitate the pregnanediol with an aqueous solution of sodium hydroxide. When working with very small amounts of pregnanediol the loss in recovery has a comparatively large effect on the accuracy of the results. Furthermore, it was found that the gravimetric determination of the precipitated pregnanediol was far more accurate than the colorimetric. Guterman's method (with some technical improvements) was highly satisfactory as regards the mode of extraction and the subsequent removal of the acidic and phenolic compounds, but for the isolation of the pregnanediol a chromatographic adsorption method was employed that allowed the very nearly complete recovery of the total amount of pregnanediol in a pure crystalline state.

METHOD

A. *Hydrolysis and extraction*

A sample of 100 ml. of urine from a 24 hr. specimen, 50 ml. toluene, and 10 ml. conc. HCl are refluxed for 15 min. The mixture is allowed to cool to room temperature and transferred to a separating funnel. The lower layer of urine is drawn off, the upper layer, consisting of toluene and emulsion is washed twice with 15 ml. of 0·1 N-NaOH and subsequently twice with 15 ml. of distilled water.

B. *Removal of phenolic and acidic compounds*

The washed solution is boiled until the water is completely removed by evaporation. After cooling the solution to 30–40°, a solution of 10 ml. 1·5% (w/v) NaOH in absolute methanol is added and the mixture evaporated until it has assumed a pale yellow to green-yellow colour. The hot toluene solution is filtered through a Pyrex glass filter (porosity G 4) with slight suction. The precipitate is washed with 15 ml. hot toluene and the combined filtrates are evaporated to dryness by heating on a hot plate in a gentle stream of air. Care must be taken not to exceed a temperature of 120° and the last traces of toluene must be removed.

C. *Isolation of pregnanediol*

To the residue 4 ml. benzene are added and the solution obtained is passed through a column (dimensions 10 × 120 mm.) of Merck alumina, standardized according to Brockmann. The column is washed twice with 3 ml. benzene and then four times with 3 ml. benzene containing 20% (v/v) ether. The crystalline product obtained by evaporation *in vacuo* of the combined four benzene-ether extracts is washed with 1 ml. absolute ether, and is weighed. In a typical experiment the product had m.p. 232–236°. After recrystallization from methanol the purified substance melted at 234–236°. Admixture with an authentic specimen did not depress the melting-point.

RESULTS

Accuracy of the method. Pregnanediol was determined in pregnancy urine and menopause urine. A second series of assays was carried out after the addition of authentic pregnanediol to the same urine specimens. The results obtained are shown in Table 1.

Table 1. *Pregnanediol determination in various urines, before and after the addition of authentic pregnanediol to the specimens*

Material	Pregnanediol (mg./100 ml.)	Pregnanediol added (mg./100 ml.)	Total pregnanediol found (mg./100 ml.)
Menopause urine	0·0	1·2	1·1
	0·0	1·0	0·9
	0·0	0·6	0·5
Pregnancy urine			
11th week	1·2	2·0	3·2
12th week	1·3	0·7	1·9
13th week	1·5	1·0	2·5
14th week	1·5	0·6	2·0

It is apparent that the method developed in our laboratory allows very nearly quantitative recovery of the pregnanediol even when only small amounts are present. A loss of about 10% is inevitable, because it is hardly practicable to elute the last traces of pregnanediol from the adsorption column.

The complete determination can be effected in 3·5 hr. Four to six tests can be made in one day by one person.

Excretion of sodium pregnanediol glucuronidate subsequent to injection of progesterone. The excretion of sodium pregnanediol glucuronidate in the urine may result from the metabolism of progesterone derived from activity of the corpus luteum, or may result from conversion of deoxycorticosterone and related compounds secreted by the adrenals. Investigations of various authors have shown that 5–11% of administered progesterone is excreted in the urine as sodium pregnanediol glucuronidate, in the human menopause (Westphal, 1940) or in normal female rabbits (Westphal, 1941, 1942*a*; Heard, Bauld & Hoffmann, 1941). Pregnanediol has also been found in the urine of normal men and hysterectomized women after administration of progesterone (Buxton & Westphal, 1939). The conversion of deoxycorticosterone into pregnanediol was established

by Westphal (1941, 1942*a*, 1942*b*). The urine of normal male and female rabbits after injection of deoxycorticosterone contained pregnanediol in amount corresponding to 7–20 % of the deoxycorticosterone administered.

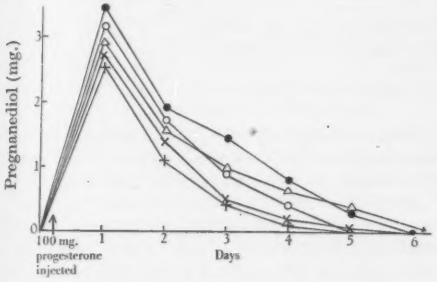

Fig. 1. Excretion of pregnanediol following the injection of 100 mg. progesterone in menopause women (5 cases).

For our purposes it was important to investigate the type of pregnanediol excretion following the injection of progesterone to menopause patients. Five normal menopause patients received each an injection of 100 mg. progesterone. Pregnanediol was determined in the five urine specimens before the injection and on each of 7–8 days after the administration of progesterone.

The graphs in Fig. 1 show that the rate of the excreted progesterone in the metabolized form of pregnanediol has a maximum level 1 day after the administration of progesterone. The rate of excretion drops to a low level during the following 5 or 6 days and the excretion of pregnanediol ceases on the 6th or 7th day. The graphs are very similar to those obtained in the urinary excretion of oestrogens after injection of oestradiol and oestrone to ovariectomized women.

SUMMARY

1. A method is described for the quantitative isolation of free pregnanediol from normal pregnancy and non-pregnancy urine of women, which allows the recovery of small amounts (0·5 mg. %).

2. Pregnanediol was determined in the urine of menopause patients following the injection of progesterone, and it is shown that the administered hormone is partially excreted as the conversion product pregnanediol, over a period of 5–6 days.

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The Mode of Action of Lipotropic Agents

PROOF OF THE *IN VIVO* INCORPORATION OF TRIETHYL-β-HYDROXYETHYL-AMMONIUM HYDROXIDE INTO THE PHOSPHOLIPID MOLECULE

By C. S. McARTHUR, C. C. LUCAS and C. H. BEST
Banting and Best Department of Medical Research, University of Toronto

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Several theories have been proposed to account for the lipotropic action of choline, betaine, methionine, inositol and other compounds which exert similar effects on the deposition of lipids in the liver. The discovery (Hershey, 1930; Hershey & Soskin, 1931) reported from the laboratory of one of us (C.H.B.) that lecithin prevents the accumulation of excessive amounts of fat in the liver of depancreatized dogs seemed to offer support for the hypothesis, originally advanced by Leathes, that fatty acid transport involves incorporation of these compounds into the phospholipid molecule. The demonstration by Best & Huntsman (1932) that choline is the lipotropically active constituent of the lecithin molecule did not alter the main concept. If choline cannot be synthesized by mammals, or if its rate of synthesis is not equal to all requirements, the amount available would probably be the limiting factor in the formation of new lecithin molecules. Thus the viewpoint has been adopted that ingested choline exerts its lipotropic effect, in part at least, by stimulating synthesis of lecithin. Using radioactive phosphorus as a tracer element, a more rapid turnover of liver phospholipids was shown to follow ingestion of choline (Perlman & Chaikoff, 1939) and the relatively rapid incorporation of dietary choline into the phospholipid molecule has been established (Stetten, 1941). Welch & Landau (1942) have shown that arsenocholine (an analogue of choline containing arsenic in place of nitrogen), which is lipotropic, also enters the lecithin molecule. The fact that inositol, which possesses lipotropic properties (Gavin & McHenry, 1941), is also a constituent of phospholipids (Anderson, 1930; Folch & Woolley, 1942) is of considerable interest to those who adopt the viewpoint that a lipotropic agent exerts its effect by virtue of promoting phospholipid synthesis. Methionine and betaine probably act by supplying labile methyl groups for choline synthesis.

About 10 years ago, before tracer elements were available, Channon & Smith (1936) first proposed testing the hypothesis that choline exerts its lipotropic action through promoting synthesis of lecithin. They wrote: 'Light should be thrown on this question if a base could be found which possessed an action on liver fat similar to that of choline and which could be shown to be present in the liver... by isolation of a suitably characteristic derivative. Such a base might be found to have been incorporated in a new phosphatide molecule in which it had replaced the choline of lecithin'. Channon & Smith (1936) described the synthesis of the ethyl homologue of choline (triethyl-β-hydroxyethylammonium hydroxide) and discovered its strong lipotropic activity. The subsequent attempt by Channon, Platt, Loach & Smith (1937) to detect the compound in liver phospholipids, after feeding it to rats for 20 days, was unsuccessful. They utilized the lesser solubility of the chloroaurate of the triethyl homologue in their search for it in the hydrolyzed liver phospholipids. Channon *et al.* (1937) stated at the end of an experimental paragraph, 'The absence of triethyl choline chloroaurate was thus established'. This wording was unfortunate since all that was actually proven was that the compound sought for was either absent or present in amounts too small to be detected by the procedure used. This failure to establish the presence of the triethyl homologue in the liver phospholipids has acted as an impediment to the general acceptance of the hypothesis that the lipotropic action of choline involves reactions which utilize the intact molecule.

The writers of this communication felt, as did Channon *et al.* (1937), that '...it is a particularly attractive hypothesis... that choline should exercise its action by enabling lecithin synthesis to occur' and in view of the work of Welch & Landau (1942) it was considered desirable to reinvestigate the ingenious attempt of Channon and his colleagues to use the triethyl homologue of choline as a tracer substance. The procedure used by Channon *et al.* (1937), which would not disclose the presence of less than 20% of the homologue when mixed with choline, did not seem to us adequate for the task in hand. We were thus led to reinvestigate the problem with new analytical and fractionation procedures.

When it was shown (McArthur, Lang & Lucas, 1945) that the *cholineless* mutant (34486) of the bread mould *Neurospora crassa* was unable to utilize the triethyl homologue of choline (cf. Horowitz, Bonner & Houlahan, 1945), and that there was a significant difference between the enneaiodide assay

for choline (Erickson, Avrin, Teague & Williams, 1940) and the value obtained by microbiological assay (Horowitz & Beadle, 1943), we were encouraged to search for an improved fractionation procedure which would clearly differentiate the trimethyl and triethyl homologues. After many preliminary trials it was found that the tertiary amines obtained by oxidative degradation of the 'choline fraction' (Lintzel & Fomin, 1931; Lintzel & Monasterio, 1931) could be separated out by fractional 'micro-distillation'. The trimethylamine and triethylamine were identified as chloroaurates.

Since the correct chemical name of the compound (triethyl-β-hydroxyethylammonium hydroxide) is a lengthy one and since there are serious objections to the use of the chemically incorrect name 'triethyl choline' we propose, for the sake of brevity, to refer to the free base as TE (i.e. the triethyl homologue) and to its chloride as TE.Cl.

It has been possible to establish unequivocally that the triethyl homologue of choline is incorporated in appreciable amount in the liver phospholipids of rats ingesting this substance (McArthur, 1946). The fact that this unnatural quaternary ammonium base, which possesses lipotropic activity, is utilized in the formation of liver 'lecithin', may be taken as support for the view that the lipotropic action of choline is dependent upon the intact choline molecule and that the natural substance acts in part at least by favouring lecithin synthesis.

EXPERIMENTAL

Four groups of rats were fed a diet containing 1·125% of TE.Cl for periods of 10, 20, 30 and 40 days, respectively. Each group consisted of 10 males and 10 females (100–170 g.). The diet had the following composition: beef dripping 25; corn oil (Mazola) 5; casein 8; gelatin 12; salts (McCollum 185) 5; agar 2; sucrose 39·9; 'vitamin powder' 2; cod-liver oil concentrate (Ayerst, McKenna & Harrison) 0·010. The 'vitamin powder' was a mixture in powdered sucrose of such a composition that at a food intake of 8 g./day the rats would receive 30 μg. aneurin hydrochloride, 25 μg. riboflavin, 20 μg. pyridoxin hydrochloride, 100 μg. calcium pantothenate and 100 μg. nicotinic acid. The diets, freshly prepared about every 5 days, were stored in tightly covered tin canisters in the refrigerator. The rats, housed in individual cages with false bottoms of coarse wire screen, were supplied daily with fresh water (*ad lib.*) and food (8·0 g.). Left-over food averaged 1·7–2·0 g. daily. From the individual food consumption the average daily intake of TE.Cl per rat for each group was calculated to be: 10-day group, 71 mg.; 20-day group, 68 mg.; 30-day group, 69 mg.; 40-day group, 69 mg. Thus the average daily intake of the substance, calculated as the base (69 mg. $\times$ 163·3/181·7 = 62·1 mg.) was nearly six times that of the rats in the experiment of Channon *et al.* (1937) (11·5 mg.). The average loss of weight of rats fed this diet for 10 days was 11·2%, for 20 days 9·2%, for 30 days 14·5% and for 40 days was only 9·1%.

The animals were stunned, the livers were removed, weighed and disintegrated immediately in a Waring blendor, under acetone, and extracted repeatedly with boiling absolute ethanol. The combined extracts were taken to dryness *in vacuo*, under N_2, at a bath temperature below 45°. The oily residue was dehydrated by repeated evaporation *in vacuo* of 50 ml. portions of benzene-absolute ethanol (4 : 1 v/v) which were added to the flask. The dry lipid material was taken up in light petroleum (b.p. 30–60°) and made up to volume (500 ml.) at about 3–4°. The solutions were kept in a refrigerated room and all samples were removed there. The analytical procedures applied to these lipids have been described recently (Best *et al.* 1946). Certain of the results are presented in Table 1.

Table 1. *Effect of feeding triethyl-β-hydroxyethylammonium chloride*

(20 rats (10 ♂, 10 ♀, average weight 138 g./group) fed test diets for periods shown. Average daily intake of TE.Cl 69 mg. Average weight loss of 9% after 40 days on diet.)

Duration of feeding TE.Cl (days)	No. of survivors	Wet wt. of livers (g.)	Total lipids (g.)	Total lipid P (mg.)	Total* choline (enneaiodide) (m.equiv.)	True* choline (*N. crassa*) (m.equiv.)	TE* (mg.)
10	18	117·5	7·135	153·1	2·45	2·11	55·5
20	20	118·9	11·66	187·5	2·62	2·37	40·9
30	18	112·9	9·38	173·5	2·60	2·08	84·9
40	19	115·6	13·36	154·2	2·54	1·91	103·0

* 'Total choline' was determined by precipitation with KI-I_2 and titration of the enneaiodide (i.e. true choline plus any other quaternary bases precipitated by this reagent), and expressed as milliequivalents. True choline, estimated by microbiological assay with the *cholineless* mutant (34486) of *Neurospora crassa*, was similarly expressed. The difference (assumed to be due to TE) was multiplied by the m.equiv. weight of TE (163·3) to give the figures in the last column. The liver fat of animals receiving no TE (i.e. basal group or groups receiving choline supplements) showed no appreciable difference (±2%) between choline assays determined chemically (enneaiodide) and microbiologically.

Isolation of liver phospholipids

The solvent was evaporated from the main portion of the light petroleum solution of the total lipid and the residue was redissolved in a small volume (10 ml.) of ether. To this solution 20 vol. of anhydrous acetone were added. The precipitation of the phospholipids was enhanced by a small addition (1·2 ml.) of saturated ethanolic $MgCl_2$ solution. The mixture, after standing overnight, was centrifuged and the supernatant solution was set aside. The phospholipid,

reprecipitated from ethereal solution with acetone and $MgCl_2$, was dissolved in moist ether and a small amount of ether-insoluble matter was removed. Both the ethereal solution of the phospholipids and the pooled acetone mother liquors were analyzed for lipid P and choline. About 3–5% of the P and about 10% of the choline (enneaiodide determination) escaped precipitation.

The remainder of the phospholipid solution was evaporated to dryness and hydrolyzed as described below (p. 615). Choline was determined in the hydrolysate by the enneaiodide procedure and also by microbiological assay. One minor modification was found advisable in the enneaiodide procedure of Erickson *et al.* (1940), viz. to use a more concentrated oxidizing reagent: 1 ml. bromine in 100 ml. glacial acetic acid containing 10% potassium acetate. On known solutions and hydrolysates of normal liver phospholipids the results obtained by the two methods were in excellent agreement.

Preliminary chemical investigation of choline homologues

A search of the literature and some preliminary investigations in this laboratory failed to reveal a suitable method of separating choline from its mono-, di- or triethyl homologues. Finally, resort was made to permanganate oxidation of these compounds to form tertiary amines which were found to be separable by distillation and then identifiable as their chloroaurates. All boiling-points and melting-points reported in this paper have been corrected.

Source of compounds required

(*a*) Trimethylamine hydrochloride (Hoffmann-La Roche) was used without further purification.

(*b*) Triethylamine (Eastman Kodak) was dried over solid KOH and fractionally distilled to yield a product boiling at 88–89°.

(*c*) Dimethylethylamine and methyldiethylamine were prepared from ethylamine and diethylamine (Eastman Kodak), respectively, by methylation with paraformaldehyde and formic acid according to the procedure of Clarke, Gillespie & Weisshaus (1933) (cf. Sommelet & Ferrand, 1924). The products thus obtained were dried over solid KOH and fractionated in a Widmer column. The dimethylethylamine (b.p. 36–37°) yielded a chloroaurate m.p. 212°. The methyldiethylamine (b.p. 65·5°) formed a chloroaurate melting at 151–152°.

(*d*) Ethylene chlorohydrin (British Drug Houses) was fractionally distilled to give a product boiling at 127–128°.

(*e*) Triethyl-β-hydroxyethylammonium chloride was prepared by heating equimolecular proportions of ethylene chlorohydrin and triethylamine in sealed tubes at 120° for 24 hr. The reaction mixture was dissolved in warm absolute ethanol (0·5 vol.) and the crystalline quaternary ammonium salt was precipitated by the addition of 1 vol. of acetone and 5 vol. of dry ether. After several recrystallizations from mixtures of absolute ethanol, acetone and ether a small portion of the product was converted to the chloroaurate, which had a satisfactory m.p. (230°).

(*f*) The other homologues of choline were prepared from the corresponding tertiary amines in a similar manner.

Oxidative degradation of choline homologues

Preliminary quantitative experiments, one of which is reported here, on the alkaline permanganate oxidation of triethyl-β-hydroxyethylammonium hydroxide and collection of the resulting triethylamine showed that the procedure is applicable to TE.

To 50 ml. of an aqueous solution containing 62·08 mg. TE.Cl in a 250 ml. Lintzel flask, 20 g. of solid NaOH were added. A slow stream of air, washed by dilute H_2SO_4, was drawn through the apparatus into a tube containing 10·26 ml. of 0·100 N-H_2SO_4 and two drops of 0·02% methyl red in 60% ethanol. The strongly alkaline solution was heated to boiling and aqueous $KMnO_4$ (0·5%) was added carefully from the thistle-tube at such a rate that no green colour, due to excess manganate, persisted in the reaction mixture until the oxidation had been completed. The heating and aeration were continued for a further 45 min. At the end of this period the flask and its contents were allowed to cool while being very slowly aerated. The contents of the receiving tube required 6·84 ml. 0·100 N-NaOH for back titration. The recovery of volatile base, subsequently shown to be triethylamine, was 100·1% of theory.

Since it had been established that the trimethyl and triethyl compounds behave identically as far as the oxidative degradation is concerned, it was assumed that the mono- and diethyl homologues would behave similarly.

Separation of tertiary amines by 'distillation'

In view of the great difference in the boiling-points of trimethylamine and triethylamine, 3·5° and 89°, respectively, it seemed feasible to separate small amounts of these by fractional distillation. Several small stills of various designs were made and tested. The most suitable of these had the form and specifications shown in Fig. 1.

The amine or mixture of amines, converted to the hydrogen sulphates or hydrochlorides, is transferred to the distilling flask (*A*) in methanolic solution and the solvent is driven off by warming the flask while a slow stream of nitrogen is passed in through tube (*E*). The receiver (*C*) is charged with 10 ml. of neutral methanol containing two drops of 0·02% methyl red indicator. The distilling flask (*A*), the U-tube (*B*) and the delivery tube (*H*) are joined through the ground-glass joints (*F*) and (*G*). The U-tube is cooled in a bath of ethanol and solid carbon dioxide and a boiling water-bath is placed around (*A*) so that not only the flask but also the side arm is kept at 100°. Through the stopcock (*K*), 2 ml. of aqueous NaOH (saturated at 35°) are run into (*A*) from the cup (*D*). The stopcock is now turned to close the opening from (*D*) and to connect (*E*) with (*A*) so that a slow stream of nitrogen may be passed through (*A*) to carry the amine into the U-tube condenser. After sweeping for 10 min. the boiling water-bath is removed from (*A*) and the ethanol-carbon dioxide bath around (*B*) is replaced by a cold water-bath which is maintained at a uniform temperature of 4° by mechanical stirring and occasional

additions of small pieces of ice. As the flask (*A*) cools, it is necessary to allow nitrogen to flow through the stopcock to prevent the methanol from being drawn over into (*B*). When (*A*) has reached room temperature, (*E*) is connected to a gasometer which is arranged to deliver a slow, constant flow of nitrogen (about 3 ml./min.). Sufficient standard acid is added from the burette (*I*) to keep the solution in (*C*) distinctly acidic and (*K*) is turned to admit nitrogen from the gasometer. When the portion of acid added initially to (*C*) has been neutralized, further known increments of the standard acid are added from the burette and the time intervals required for the neutralization of these by the amine carried over are recorded.

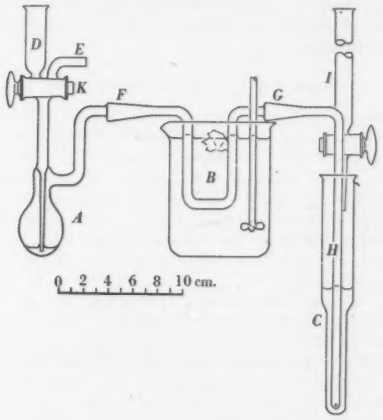

Fig. 1. Diagram of apparatus used for the separation of the tertiary amines.

Typical curves obtained with the four possible tertiary amines, viz. trimethylamine, dimethylethylamine, methyldiethylamine and triethylamine, and a known mixture of the first compound with the last, are shown in Fig. 2. Curve 1 shows that trimethylamine (80·0 mg.) 'distils' at 4° with great rapidity, the bulk of it going over in about 3 min. The relatively slow evolution of triethylamine (20·0 mg.) is apparent in curve 5, while curves 3 and 4 indicate the rates of evaporation of dimethylethylamine (101·5 mg.) and methyldiethylamine (118·4 mg.) respectively, under the same conditions. If the rate of sweeping is kept constant from one experiment to another, the amount of triethylamine present does not affect the rate of evolution, as is shown in curve 6, which represents the rate of evaporation of 40 and 80 mg. of triethylamine with slower aeration (1·8 ml. N_2/min.) than that used in obtaining curve 5. Curve 2 represents the behaviour of a mixture of 80 mg. of trimethylamine and 20 mg. of triethylamine under the standard conditions (3·1 ml. N_2/min.) used to obtain curves 1 and 5 and in all subsequent work. Since the slope of the curve for evaporation of triethylamine is constant with a constant sweeping rate, projection of the upper linear part of curve 2 should cut the vertical axis at a point representing the volume of standard acid required to neutralize the trimethylamine. The difference between this value and that of the total titration should, therefore, represent the amount of acid consumed by triethylamine. The agreement between theory and practice is satisfactory (see also Fig. 4)

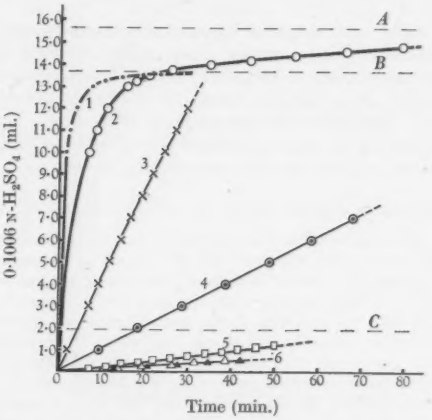

Fig. 2. Time-'distillation' curves for known substances at 4°. Sweeping rate 3·1 ml./min. for curves 1–5, 1·8 ml./min. for curve 6. Curve 1, 80 mg. trimethylamine; curve 2, a mixture of 80 mg. of trimethylamine and 20 mg. triethylamine; curve 3, 101·5 mg. dimethylethylamine; curve 4, 118·4 mg. methyldiethylamine; curve 5, 20 mg. triethylamine; curve 6, △—△ 40 mg. triethylamine, ▲—▲ 80 mg. triethylamine. Lines *A*, *B* and *C* cut the vertical axis at points representing the calculated titration values for the mixture of 80 mg. trimethyl- and 20 mg. of triethylamine, 80 mg. of trimethylamine alone and 20 mg. triethylamine alone, respectively.

Examination of the rat liver phospholipids

Hydrolysis. The phospholipid fractions from each of the four groups of animals were treated separately. Each was dissolved in 10 ml. of warm ethanol and emulsified by forcing the solution in a thin stream into 100 ml. of 5 % (v/v) aqueous H_2SO_4. The mixture was refluxed for 8 hr. After removal of the fatty acids by shaking several times with ether and precipitation of the bulk of the sulphuric acid with baryta the hydrolysate was neutralized to phenol red by the addition of 0·1 N-NaOH and made up to a volume of 500 ml.

Choline determinations in the hydrolysates. Portions of each of the four hydrolysates obtained from the acetone-insoluble phospholipids were analyzed in replicate for choline by the chemical (enneaiodide) and microbiological (*Neurospora crassa, cholineless*) methods. The results are tabulated in Table 2.

Table 2. *Choline content of hydrolysates of isolated liver phospholipids*

(Results expressed as mg./500 ml. of hydrolysate.)

Days of feeding	Chemical procedure (calculated as choline)	Microbiological procedure (calculated as choline)	Difference (calculated as TE)	TE (as % 'total choline')
10	249	216	44·4	17·1
20	263	242	28·3	10·5
30	246	202	59·3	22·7
40	254	194	80·8	29·4

Since the chemical procedure estimates total quaternary ammonium base while the microbiological method responds only to choline (and to a very much smaller extent to the methyldiethyl and dimethylethyl homologues of choline), any difference in assay was tentatively attributed to the presence of the triethyl homologue.

Oxidative degradation of phospholipid hydrolysates

40-*day group*. The portion (350 ml.) of the hydrolysate which remained after removal of samples for analytical purposes was oxidized directly with alkaline permanganate in the Lintzel apparatus as described above except that just sufficient standard H_2SO_4 was added to the receiver during the oxidation to keep the distillate acidic to methyl red. Because of the limited capacity of the reaction flask, the operation was performed on three separate portions. To the combined distillates containing the tertiary amines another volume of standard H_2SO_4, equal to that required for neutralization, was added and the acidic solution was set aside for further investigation.

30-*day group*. Because oxidation of the unfractionated hydrolysate (above) gave rise to ammonia (from hydrolytic products of cephalins) as well as tertiary amines and further because the volume of permanganate solution required was so great, it was deemed advisable to isolate the quaternary bases for oxidation. The remainder of this hydrolysate (470 ml.), made alkaline to phenol red, was concentrated *in vacuo* to about 140 ml. (bath temperature 60–70°). No volatile base was detectable in the distillate proving that the isolated phospholipid did not contain any free tertiary amine. From the concentrated hydrolysate, again made slightly acidic to phenol red, the quaternary ammonium bases were precipitated in centrifuge pots at 0° as enneaiodides. After centrifuging, the precipitate was washed once in the pots with cold potassium triiodide reagent diluted to the same concentration as was used for precipitation. The precipitate was transferred to a distillation flask with 100 ml. of water and 5 ml. N-H_2SO_4. The elemental iodine was removed by distillation under reduced pressure. Ionic iodine was precipitated with an excess of Ag_2SO_4 and the excess silver ions were removed by H_2S. The filtrate from the silver sulphide, concentrated to a volume of 30 ml., was oxidized in the Lintzel apparatus. After the addition of a second equivalent of standard H_2SO_4 to the distillate, it was set aside.

Combined 10- *and* 20-*day groups*. The remaining portions of the phospholipid hydrolysates from the 10- and 20-day groups of animals, each 450 ml. in volume, were combined and concentrated to a volume of 200 ml. The quaternary ammonium bases were isolated as described above, except that the enneaiodide was washed twice by dispersing the precipitate in diluted potassium triiodide reagent. The isolated base in aqueous solution was made up to a volume of 100 ml. and 98 ml. of this was converted to tertiary amines.

Separation of the tertiary amines

40-*day group*. The aqueous solution obtained by titration of the volatile base formed by oxidation of the hydrolyzed phospholipids was concentrated *in vacuo*. The residue was dehydrated by repeated distillation of 5 ml. portions of absolute ethanol which were added to the distillation flask. Two 5 ml. portions of absolute ethanol were used to extract the amine salts from the contaminating $(NH_4)_2SO_4$. The ethanolic extracts were taken to dryness in the micro-distillation flask.

The amines, liberated and 'redistilled' as previously described to obtain the time-'distillation' curve, required a total of 12·95 ml. of 0·1006 N-H_2SO_4. The slope of the upper linear part of the curve indicated the presence of triethylamine. The acid required by this compound corresponded to 29·0 mg. of the base. (This value agrees moderately well with that calculated from the difference between the two choline analyses, 26·6 mg.) In order to obtain a sample of this material contaminated with as little trimethylamine as possible, the contents of the receiver were fractionally 'distilled' under the same conditions. The point at which to change the receiver was determined by inspection of the time-'distillation' curve. The cut was not made until the slow constant evolution of the higher boiling amine was evident. Momentarily, the flow of nitrogen was stopped while the receiver was replaced by another containing 15 ml. of 0·1 N-methanolic HCl. Hot water-baths were placed around the distillation flask and the U-tube and sweeping was resumed at a gradually increasing rate (4–8 ml./min.). Complete transfer required about 2 hr.

The mean molecular weight of the amine mixture was determined upon the crystalline residue obtained by evaporating the acidic absorbing liquid to dryness. The residue, washed with absolute ether and desiccated (21·5 mg.) was returned to the micro-still. The volatile amines required 16·00 ml. of 0·0100 N-H_2SO_4 (methyl red indicator). The mean molecular weight (134·4) suggested that the mixture consisted of 92·4% triethylammonium chloride and 7·6% trimethylammonium chloride.

The crude triethylamine, 'redistilled' into an excess of HCl to free it from indicator, was converted to the chloroaurate in 0·5 ml. of N-HCl. The precipitate, filtered and washed twice with 0·12 ml. portions of cold dilute HCl, when dried *in vacuo* over P_2O_5 weighed 16·8 mg., m.p. 86–87°. Analysis: C, 16·5; H, 3·68; Au, 44·2%. The com-

positions and m.p. of the possible trialkylammonium chloroaurates are tabulated below:

	m.p.	C (%)	H (%)	Au (%)
$(C_2H_5)_3N.HCl.AuCl_3$	89°	16·35	3·66	44·7
$(C_2H_5)_2CH_3N.HCl.AuCl_3$	151–152°	14·06	3·30	46·2
$C_2H_5(CH_3)_2N.HCl.AuCl_3$	212°	11·63	2·93	47·7
$(CH_3)_3N.HCl.AuCl_3$	234–235°	9·03	2·53	49·4

30-day group. The acidified distillate obtained by Lintzel oxidation of the isolated choline was evaporated to dryness and the residue was transferred to the still. The time-'distillation' curve shown in Fig. 3 indicated the presence of

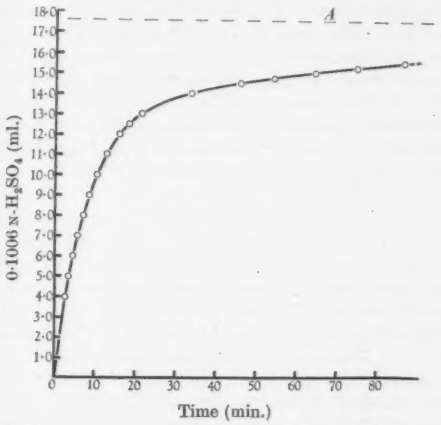

Fig. 3. Time-'distillation' curve for tertiary amine mixture derived from liver phospholipids of 30-day group. Aeration rate 3·1 ml./min. The line *A* cuts the vertical axis at a point representing the total titration.

triethylamine. From the amine mixture, fractionated as described in the previous section, the higher boiling fraction was separated. It was converted to the chloroaurate (81 mg.) m.p. 87°. Mixed with triethylammonium chloroaurate, m.p. 89°.

Combined 10- and 20-day groups. All operations upon the combined material from these two groups were conducted with quantitative care in order to compare the recoveries obtained by oxidative degradation and fractional 'distillation' with the results of the choline analyses on the bases isolated as enneaiodides. The tertiary ammonium salts were transferred to the micro-distillation apparatus. Since the amount of trimethylamine derivable from the choline in the mixture was large (30·2 ml. 0·1006 N-acid, calculated from the results of the microbiological choline assay), the U-tube was kept at a temperature of 4° for 1 hr. without aeration to avoid too rapid transfer and possible incomplete absorption of trimethylamine. During this time amine equivalent to 13·35 ml. of the standard acid was collected in the receiver, which then was replaced by a second receiver. 'Distillation' and titration of the remaining part of the amine mixture were carried out in the manner previously described. Fig. 4 shows the 'distillation' curve obtained. The total titration amounted to 32·85 ml. of the standard acid (calculated requirement from the results of the chemical analyses, 32·30 ml.). The curve (Fig. 4) indicates that the mixture consisted of 3·05 m.equiv. of trimethylamine and 0·25 m.equiv. of triethylamine; by calculation (from the results of the microbiological and chemical choline assays), the values were 3·03 m.equiv. and 0·19 m.equiv. respectively.

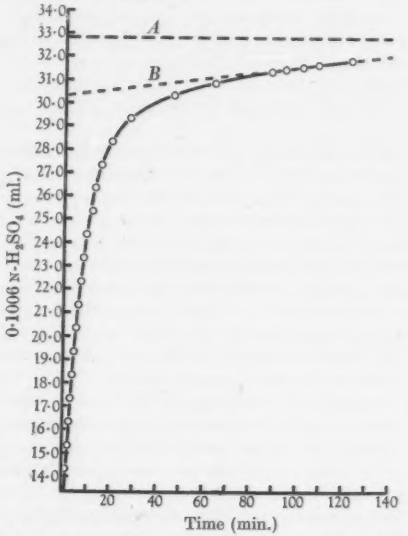

Fig. 4. Time-'distillation' curve of tertiary amines derived from liver phospholipids (10- and 20-day groups). The choline fractions from the two groups were combined for oxidative degradation and fractionation of the tertiary amines. Intercept on the vertical axis of line *A* represents the total titration of the amine mixture, of line *B* (a projection of the upper linear portion of the curve) represents the titration value for trimethylamine; the difference represents the titration value for triethylamine.

After drying, the residue from the second receiver was transferred to the micro-apparatus for fractionation. The 'distillation' was carried out with sweeping until 17·0 ml. of 0·1006 N-acid had been consumed. At this point the receiver was exchanged for one containing 20 ml. of methanol and 0·2 ml. of conc. HCl, into which the remainder of the amine was swept. The residue remaining after the removal of the methanol was converted to the chloroaurate in dilute HCl (1·9 ml.). The yellow crystalline precipitate, washed with three 1 ml. portions of cold dilute HCl and dried (33·4 mg.) melted at 89°, mixed with methyldiethylammonium chloroaurate, m.p. 124°; mixed with authentic triethylammonium chloroaurate, m.p. 89°. A further quantity (23·5 mg., m.p. 87–89°) was obtained by concentration of the mother liquors.

DISCUSSION

Although the lipotropic effect of choline is well established and generally accepted, its mode of action has not yet been completely elucidated. Some

authorities believe that the lipotropic effect may be explained by the observation that choline accelerates the turnover of the phosphorus in liver phospholipids. The assumption has been made that choline similarly promotes an accelerated turnover of all constituents of the phospholipid molecule. Experimental evidence for any increased rate of turnover of fatty acids in liver phospholipids under the influence of dietary choline, is not yet available, however, and hence a completely satisfactory explanation of lipotropic action must await further investigation.

There seems to be some confusion in the minds of certain recent writers on the subject of lipotropic action as to whether the labile methyl groups or choline *per se* is the ultimate lipotropic factor. The evidence is accumulating that the intact choline molecule is the effective lipotropic substance. Compounds such as betaine and methionine appear to exert their lipotropic effect by contributing labile methyl groups either for the synthesis or sparing of choline. The experiments of Welch & Landau (1942) have established that arsenocholine, which is lipotropic and does not possess labile methyl groups, when fed, is incorporated *in toto* into the phospholipid molecule. In the case of this choline analogue, it is the intact molecule and not a labile methyl group which exerts the biological effect.

The present experiments with the triethyl homologue of choline, which also is lipotropic and lacks labile methyl groups, supply another example of a lipotropically active substance which is utilized intact in the synthesis of new phospholipid molecules. The arguments advanced by Welch & Landau (1942) in support of the hypothesis that the intact choline molecule is the active lipotropic substance are substantiated by the findings reported above.

Several explanations may be offered for the failure of Channon *et al.* (1937) to detect the triethyl homologue of choline in the liver phospholipids of their experimental animals. The basal diet fed by them was so low in protein (5% casein) that it probably did not permit metabolic processes to function normally. At this low protein intake, the quaternary ammonium chloride may have exerted a more toxic action than it did in the present experiments. Whatever the explanation, it has been possible to feed six times the daily dose used by them, and to give it for twice as long a period of time, without any greater weight loss or mortality than they observed. Furthermore, improvements in micro-analytical methods during the past 10 years have probably contributed to the present successful accomplishment of the project initiated by Channon and his colleagues. The establishment of the fact that the lipotropically active triethyl homologue of choline is biologically incorporated into the molecule of liver phospholipids, removes one of the impediments to general acceptance of the hypothesis that the lipotropic action of choline involves reactions which utilize the intact molecule.

SUMMARY

1. A significant difference between the microchemical and microbiological assays for choline in the hydrolysates of the phospholipids isolated from the livers of rats fed triethyl-β-hydroxyethylammonium chloride, suggested that appreciable amounts of an unnatural base had been incorporated into the liver phospholipids. Fractionation procedures were devised which led to chemical proof that the triethyl homologue of choline had been incorporated into the phospholipids.

2. This finding provides further proof that the lipotropic effect of choline is associated with the intact molecule rather than its methyl groups.

We are indebted to Dr J. H. Ridout for the extraction of the liver lipids and some of the preliminary chemical analyses and to Miss J. M. Lang who performed the microbiological assays. The expenses of this investigation were defrayed, in part, by a grant from the Banting Research Foundation.

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The Pigment of the Malarial Parasites *Plasmodium knowlesi* and *Plasmodium gallinaceum*

By C. RIMINGTON and J. D. FULTON, with the assistance of H. SHEINMAN
Department of Chemical Pathology, University College Hospital Medical School and Medical Research Council Farm Laboratories, Mill Hill

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The pigment produced by different malarial parasites has been extensively studied, the latest contributions relative to its chemical nature being those of Sinton & Ghosh (1934 *a*, *b*), Ghosh & Sinton (1934), Ghosh & Nath (1934), Devine & Fulton (1941, 1942) and Morrison & Anderson (1942). This aspect of the question is summarized historically in the first mentioned publication in which a close similarity if not identity was established between the pigment and haematin prepared from crystalline haemin and alkali. Ghosh & Sinton (1934) describe (without illustrations) the preparation of crystals of pyridine haemochromogen and of haemin from washed pigmented *P. knowlesi* parasites. The same crystalline derivatives (unillustrated) were obtained by Morrison & Anderson (1942) using portions of the parasite mass. Devine & Fulton (1941, 1942) present more extended spectroscopic data in support of the identity of *P. knowlesi* and *P. gallinaceum* pigment with haematin, including an examination in the visible region of met- and fluoromethaemoglobins, and oxy and reduced haemoglobins prepared by combining the amorphous malarial pigment with native ox globin. No measurements of the Soret bands were performed. Heilmeyer (1943), from a spectrophotometric study, has shown that all preparations of malarial pigment available to him contained variable amounts of contaminating substances of high molecular weight.

All the above workers relied upon dilute alkali (Na_2CO_3 or NaOH) to extract the pigment from the parasites, and crystalline derivatives, where described, were formed only from the crude parasite mass before such extraction. It is well known that dilute NaOH frequently so alters haematin during the process of dissolution and extraction from natural sources that it is subsequently well-nigh impossible to prepare crystalline derivatives (Fischer & Schneller, 1923). Keilin (1943) has also recorded the rapid change in properties brought about by the action of dilute Na_2CO_3 upon protohaemin.

Having available a new technique for the extraction of haematin without at any stage using alkalis, a method successfully applied by Schwabacher, Lucas & Rimington (1947) to the isolation and identification of haematin from *Fusiformis melaninogenicus*, it seemed desirable to re-examine malarial pigment from different sources and to prepare crystalline derivatives for identification from the isolated pigment, thus excluding any possibility that these were derived from a cellular material of the parasite other than the pigment itself. Moreover, the identification of this substance with haematin (ferriprotoporphyrin) depends, according to published work, upon comparison of the spectrophotometric absorption curves, usually obtained with the use of serial filters, of the pigment and haematin in dilute NaOH. These curves show only broad maxima of low intensity and the agreement in some cases is far from good. The absorption curves for the acid acetone solutions of malarial pigment and of haematin from haemoglobin, published by Morrison & Anderson (1942), show general similarity but not identity and examination is not recorded beyond 500 mμ. No author, so far as we are aware, has seriously considered the possibility that the vinyl groups of the protoporphyrin nucleus might be reduced by the activity of the parasite to form mesohaematin or even eliminated entirely, with the resultant production of deuterohaematin. That such changes may be brought about by micro-organisms is evident from the detection of both meso- and deuteroporphyrins in faeces by Zeile & Rau (1937). In the present study particular attention has been paid to this point.

EXPERIMENTAL

Pigment from P. knowlesi. Malarial parasites (Devine & Fulton, 1941) from a *P. knowlesi* infection of monkeys were used. After thorough washing, the parasite mass was stirred with 90 % phenol solution, prepared by adding 10 ml. water to 90 g. phenol, and extraction allowed to proceed at room temperature with occasional stirring for 12 hr. The mixture was then centrifuged and the residue re-extracted with successive amounts of the phenol solution at 2–3 hr. intervals until an absorption band at 627 mμ. was no longer visible in the extract. To the pooled solutions of phenol, 2 vol. of ethanol were then added to precipitate small quantities of dissolved protein and after filtration through a no. 4

sintered glass funnel the solution was dialyzed against tap water in a stout 'Visking' bag, the contents being frequently mixed as two phases rapidly separated. Dialysis was finally continued against distilled water and as soon as turbidity or precipitation was noticed in the lower, pigmented layer, the latter was removed into a centrifuge tube and diluted liberally with water, whereupon the pigment separated in a granular form. It was collected on the centrifuge, washed repeatedly with water and then dried *in vacuo*.

The pigment is obtained at this stage as an amorphous black powder almost completely soluble in pyridine-chloroform mixture (3:8) but insoluble in ether or ethanol; acid ether or acid acetone, however, dissolve it readily. That such preparations are free from protein is demonstrated by the absence of any haemochromogen spectrum when sodium hyposulphite (sodium hydrosulphite, $Na_2S_2O_4$) is added to a solution in dilute NaOH. Small amounts of impurity were removed and crystallization was effected as follows. For every 50 mg. of pigment was added 0·15 ml. of pyridine, then 0·4 ml. of chloroform and the very concentrated solution was filtered through a small sintered glass funnel; the filtrate was then added drop by drop to 2·5 ml. of acetic acid-chloride mixture (25 ml. acetic acid + 0·5 ml. saturated NaCl + 0·4 ml. conc. HCl) maintained in a small tube at boiling water-bath temperature. After 0·5 hr. the mixture was allowed to cool and placed in the ice chest overnight. The crystals were collected and washed on the centrifuge by 50 % (v/v) acetic acid, water, ethanol and finally ether. Recrystallization was effected in the same manner (Fig. 1).

Pigment from P. gallinaceum. For this preparation, blood from parasitized fowls was used (Devine & Fulton, 1942) and the method of extraction differed in no way from that described above. Extraction of the pigment by phenol was, however, slower and a considerable proportion of the crude material was insoluble in pyridine-chloroform, no doubt owing to the impossibility in this case of separating the parasites from erythrocyte debris. This impurity was not protein in nature, it was only sparingly soluble in sodium hydroxide solution and was not further investigated. One recrystallization afforded well-formed crystals of the pigment (Fig. 2). Haemin from blood, similarly recrystallized, is shown for comparison in Fig. 3.

That the malarial pigment derivative which closely resembled protohaemin in appearance, was in fact a haem pigment, was readily demonstrated by spectroscopic examination of its solutions in different solvents (Hartridge reversion spectroscope), by the formation of a pyridine haemochromogen, of a porphyrin when the pigment was treated with concentrated sulphuric acid and of methaemoglobin (and thence HbO_2, Hb and HbCO)* when it was combined in faintly alkaline solution with native ox globin (Tables 1 and 2).

Table 1. *Absorption maxima shown by malarial pigment and by crystalline haemin*

Solvent, treatment, etc.	Malarial pigment (mμ.)	Haemin (mμ.)
0·1 N-NaOH	615·4	615·7
Above + $Na_2S_2O_4$	*c.* 583–535	575–540
Above + CO	569·3 533·9	565·0 536·0
0·1 N-NaOH + pyridine (10 % by vol.) + $Na_2S_2O_4$	556·6 528·3	556·3 528·0
90 % phenol	627·4 540·3	627·6 540·0
Ether: HCl	637·1 543·6	637·6 543·7
Ether: acetic acid	634·6 542·9	633·1 541·4
Conc. H_2SO_4	601·9 557·7	601·7 556·6
Porphyrin from above in pyridine	623·6 576·3 535·6 505·3	625·8 579·3 540·3 507·8
Haematoporphyrin in H_2SO_4	— —	601·6 554·3
Mesoporphyrin in H_2SO_4	— — —	594·5 556·9 551·9

Table 2. *Absorption maxima shown by reconstituted haem-ox globin pigments*

Pigment type	Malarial pigment-ox globin (mμ.)	Haematin-ox globin (mμ.)
Met Hb	630·9	631·8
HbO_2	576·3 539·4	577·7 539·4
HbCO	572·1 537·4	572·1 537·8
	Soret bands	
HbO_2	405·5	405·8
HbCO	416·5	416·0

On mixing a small portion of the pigment on a glass slide with Takayama's reagent (Harrison, 1944), it dissolved with a red colour and under the microscope crystal masses of pyridine haemochromogen were visible. The shape of these crystals, even when prepared from pure haematin, is variable, ranging from prismatic needles grouped in stellate

* For the measurement of the Soret bands of these pigments we are indebted to Mr E. M. Jope of the M.R.C. Spectroscopy Unit, London Hospital, who used a Hilger medium quartz spectrograph.

Fig. 2. Haemin from pigment of *P. gallinaceum*. ×540.

Fig. 1. Haemin from pigment of *P. knowlesi*. ×540.

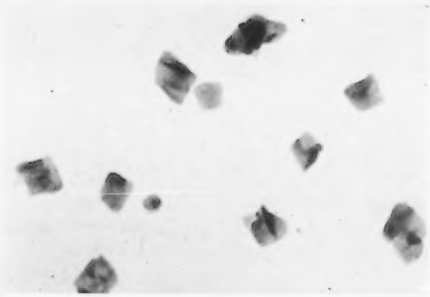

Fig. 5. Pyridine haemochromogen from haemin. ×875.

Fig. 4. Pyridine haemochromogen from malarial pigment. ×875.

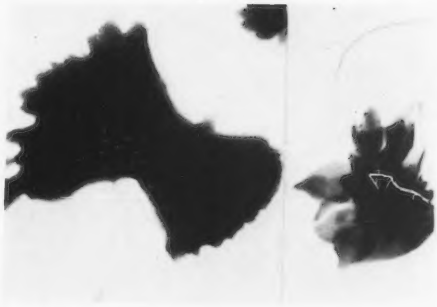

Fig. 3. Haemin. ×540.

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clusters to flat, overlapping platelike structures. It was observed that the type of crystal produced depended somewhat upon the maturity of the Takayama's reagent and hence the velocity of reduction and also that needles first formed tended to become transformed on the slide into plates. Control experiments were therefore always done at the same time with pure crystalline haemin (see Figs. 4 and 5).

The preparation of the reconstituted methaemoglobin, etc. was accomplished as follows:

Globin hydrochloride was prepared from ox blood (ox globin being relatively more stable than horse or human globin) and partially renatured by dialysis for 24 hr. against running tap water according to the method of Anson & Mirsky (1929–30). Dialysis was completed against M/30-K_2HPO_4 solution at the pH of which denatured globin is insoluble. After centrifugation, the supernatant liquid containing the renatured protein was carefully adjusted by KH_2PO_4 solution to pH *c.* 6·0. To separate portions of this solution were added solutions in dilute NaOH of malarial pigment and of pure crystalline haemin from blood (Rimington, 1942) respectively until the band of methaemoglobin at about 630 mμ. was clearly visible. After this was measured, freshly prepared Stokes reagent (Harrison, 1944) was added drop by drop until the colour changed from brown to red and the methaemoglobin spectrum had given place to that of oxyhaemoglobin. Over-reduction led to the single, broad band of reduced haemoglobin (purple coloured solution), but shaking with air restored the two-banded spectrum of oxyhaemoglobin. This demonstrated, incidentally, the capacity of the synthetic globin-pigment complex to function as an oxygen carrier as does haemoglobin.

It was observed that during the addition of the Stokes reagent some unavoidable denaturation occurred, so the solutions were centrifuged before proceeding further from this stage. For measurement of the Soret bands, the reconstituted haemoglobins were equilibrated to pH 7·2 by dialysis against M/30 phosphate buffer, since the exact position of this maximum is influenced by the pH of the solution. Good agreement between the reconstituted malarial pigment and that from haemin was found for both oxy and carboxy derivatives, the intensity of the Soret bands being of the order of ten times that of the visible bands as is the case with normal native oxyhaemoglobin. The actual maxima of the Soret bands were in each case at a wavelength shorter than that usually observed in the case of native oxyhaemoglobin and carboxyhaemoglobin, 405·5 mμ. as compared with the usual 414·5 mμ. for oxyhaemoglobin and 416·5 mμ. as compared with the usual 420·0 mμ. for carboxyhaemoglobin.

These shorter wave-lengths are those observed for the Soret bands on solutions of normal native oxyhaemoglobin and carboxyhaemoglobin respectively which have been kept for some time and have apparently undergone some preliminary form of denaturation, which does not, however, appear to affect the visible band wave-lengths. In the case of the reconstituted malarial pigments, the visible bands agree closely with those of normal native oxyhaemoglobin and carboxyhaemoglobin. The wave-lengths observed for the Soret band in these reduced reconstituted pigments and in partially denatured haemoglobins almost coincide with that of methaemoglobin. The presence of a Soret band is in itself strong evidence for the existence of the intact porphyrin ring.

DISCUSSION

The close correspondence of the absorption spectra of the malarial pigment with haematin and the porphyrin derived from it with haematoporphyrin rather than mesoporphyrin (see Table 1) or deuteroporphyrin indicates that malarial pigment from both sources is indeed haematin in which the vinyl groups have remained intact. Drabkin (1942) has shown that the presence of free vinyl groups in haem pigments displaces their absorption maxima about 11 mμ. towards the red, the α band of pyridine haemochromogen, for example, lying at 558 mμ. and that of pyridine mesohaemochromogen at 547 mμ. Further evidence of the identity of each pigment with haematin (ferriprotoporphyrin) is afforded by comparison of the spectral absorption in the visible and ultra-violet region of the haemoglobins and their derivatives made by combining these pigments with renatured ox globin.

SUMMARY

1. The pigment of malarial parasites (*P. knowlesi* and *P. gallinaceum*) has been extracted by a technique which avoids the use of alkali at all stages.

2. From this material, crystalline haemin and pyridine haemochromogen have been prepared.

3. A comparison of the spectral absorption of malarial pigment and of haemin in different solvents and of the visible bands and Soret bands of haemoglobin derivatives made by combining these pigments with renatured ox globin confirms the identity of both malarial pigments with haematin (ferriprotoporphyrin).

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Acetylation and Deacetylation of the *p*-Amino Group of Sulphonamide Drugs in Animal Tissues

By H. A. KREBS, W. O. SYKES and W. C. BARTLEY, *The Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield*

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The work reported in this paper was originally intended to deal with the mechanism of the acetylation of sulphonamides in animal tissues, with special reference to the source of the acetyl radical and the conditions controlling the rate and degree of acetylation. We thought it would be advantageous to use for this work isolated tissues, sliced or minced, but soon found that the degree of acetylation in isolated tissues was much lower than was to be expected on the basis of observations on the intact organism. Whilst in the intact guinea-pig, for example, 75% of the sulphamezathine excreted in the urine was conjugated, conjugation by slices of guinea-pig tissues never exceeded 20% and was usually below 10%, even when the added amount of drug was small. Such acetylation as did occur in isolated tissues was usually completed within 30 min. of incubation. An investigation of this apparent discrepancy in the metabolic behaviour of tissues *in situ* and *in vitro* occupies the main part of this paper. At least a partial explanation for the discrepancy offered itself when it was found that certain tissues contain an enzyme as already reported by Kohl & Flynn (1940) which hydrolyzes N^4-acetylated (or, generally, N^4-acylated) sulphonamides. It appears that the relative activity of this enzyme is altered by slicing or mincing. The activity is apparently low, perhaps even negligible, in the intact rabbit or guinea-pig, though it is operative in the intact pigeon, dog and cat. The enzyme activity is increased, either in absolute terms or relative to conjugating systems, by the conditions prevailing in the *in vitro* experiments. *In vitro*, therefore, the process of acetylation appears to be largely counterbalanced by reactions causing deacetylation.

EXPERIMENTAL

Determination of sulphonamides

The methods of Bratton & Marshall (1939) and of Rose & Bevan (1944) were used.

Blood. A suitable volume, usually 0·1 ml., was measured into 0·5 ml. of 0·5% saponin solution in a 10 ml. measuring tube. Water was added to make up to 9 ml. and 1 ml. 15% trichloroacetic acid solution was used for deproteinization. One 4 ml. portion of the filtrate was used for the determination of the free sulphonamide and another 4 ml. portion for the determination of total sulphonamide. A visual colorimeter was used; the standard contained 0·02 mg. of the sulphonamide. When total sulphonamide was determined the corresponding acetyl compound, treated in the same way as the unknown, was used as a standard.

Urine. Usually 0·1 ml. of urine, if necessary diluted, was used and treated in the same way as blood, except that the saponin was left out.

Faeces. Faeces were mixed with 10 vol. of water. A sample was deproteinized with trichloroacetic acid and the filtrate treated in the same way as the blood filtrate.

Tissue extracts. Media in which tissues had been suspended or extracts of tissues were treated in the same way

as blood, except that the quantity varied according to the sulphonamide concentration. When practicable the amount of material was so chosen as to contain about 0·02 mg. of sulphonamide.

Blanks. Urines from persons or animals who had not received sulphonamides usually gave a pink-orange colour when total sulphonamide was determined, corresponding to 0·2–0·5 mg./100 ml., suggesting that there are primary aromatic amines or amine-forming substances present in normal urine. Very low concentrations of sulphonamides, therefore, cannot be determined accurately in urine. In faeces, similar difficulties were encountered. In addition, colouring matter present in the faeces interfered with the colorimetry. No satisfactory method for the removal of the interfering substance was found. Charcoal removed the coloured substances in the faeces, but also much of the sulphonamides. Owing to the presence of interfering substances, the results recorded in the paper for faeces tend to be a little too high.

Special points. When trichloroacetic acid was replaced by metaphosphoric acid, satisfactory results were obtained with sulphamezathine and a few other sulphonamides, but in some cases, especially that of sulphathiazole, the dye formed on coupling proved to be unstable; it faded within a few minutes.

N^4-Acylated sulphonamides are slowly hydrolyzed at room temperature in the trichloroacetic acid filtrate (Table 1, see also Poth & Ross, 1944). The free sulphonamides were therefore determined at once after filtration, or, if that was not possible, the filtrate was neutralized for storing. In the latter case, trichloroacetic acid was again added before the determination.

Table 1. *Hydrolysis of N^4-acetylsulphonamides in trichloroacetic acid solution*

(Acetylsulphonamide (2 mg.) in trichloroacetic solution (10 ml.); room temperature (15–20°).)

Drug	Quantity hydrolyzed (mg.) after 18 hr.	76 hr.
N^4-Acetylsulphamezathine	0·12	0·33
N^4-Acetylsulphapyridine	0·13	0·32
N^4-Acetylsulphadiazine	0·12	0·38
N^4-Acetylsulphathiazole	0·12	0·24
N^4-Acetylsulphanilamide	0·13	0·36

The term 'free' sulphonamide in this paper refers to compounds whose *p*-amino group is free; 'conjugated' refers to compounds whose *p*-amino group is set free on acid hydrolysis. The quantities of total or conjugated sulphonamides, when expressed in milligrammes, are given in terms of the free sulphonamides.

RESULTS

Experiments on the intact organism

Man. Two examples of experiments on the conjugation of sulphamezathine in man are recorded in Table 2. After a single intramuscular dose the blood concentration of the 'free' drug quickly reached a

Table 2. *Acetylation of sulphamezathine in the human body*

Blood analysis: Time after dosing (hr.)	(min.)	Sulphamezathine concentrations (mg./100 ml.) Free	Total	Ratio: Free/Conjugated	Urine analysis: Time after dosing (hr.)	(min.)	Volume excreted (ml.)	Sulphamezathine concentrations (mg./100 ml.) Free	Total	Ratio: Free/Conjugated	Total recovered sulphamezathine (mg.) Free	Total
Exp. 1. Male age 23; 70 kg. 2 g. sulphamezathine intramuscularly. (33% solution of sodium salt.)												
0	40	7·1	7·1	—	3	10	257	34·5	138	0·35	88·7	356
2	10	6·1	8·9	2·2	8	55	285	30·0	287	0·12	174	1173
3	55	4·7	7·5	1·7	10	0	205	4·6	65·5	0·076	184	1308
5	0	3·9	7·3	1·15	11	30	96	8·4	145	0·061	192	1446
6	40	3·1	6·2	1·0	14	40	218	4·9	110	0·047	202	1690
8	10	2·2	5·1	0·76	23	55	443	2·4	55·2	0·045	213	1930
—	—	—	—	—	27	20	305	0·8	15·1	0·056	215	1980
—	—	—	—	—	31	40	338	0·5	6·3	—	217	2000
—	—	—	—	—	33	20	155	0·4	3·8	—	218	2005
—	—	—	—	—	35	20	200	0·4	2·9	—	219	2010
Exp. 2. Male age 24; 70 kg. 1·71 g. sulphamezathine taken orally.												
0	40	1·3	1·7	3	2	30	206	7·0	38·4	0·22	14·4	79·4
1	10	2·8	3·3	6	4	50	250	16·5	105	0·19	55·7	341
1	50	3·8	4·9	3·5	7	20	288	12·5	100	0·14	91·7	631
2	20	4·1	5·3	3·4	9	25	243	8·6	102	0·092	113	898
3	25	4·5	5·5	4·5	11	15	270	5·0	68·6	0·079	126	1083
4	10	3·5	5·4	1·84	15	20	160	8·2	149	0·058	139	1322
5	55	3·0	4·8	1·67	23	05	275	3·7	83·4	0·046	150	1554
7	45	1·6	3·6	0·80	26	40	267	1·1	20·2	0·058	152	1610
9	45	1·3	3·1	0·72	28	20	150	1·0	11·7	0·093	154	1625
12	45	—	2·3	—	30	30	555	0·4	4·3	—	156	1650
—	—	—	—	—	33	20	190	0·5	6·0	—	160	1660
—	—	—	—	—	35	20	145	—	0·9	—	—	—
—	—	—	—	—	36	50	192	0·8	8·1	—	—	—

Table 3. *Fate of acetylsulphamezathine in the human body*

(Both experiments were carried out on the same male person, age 24; 70 kg. 2·30 g. acetylsulphamezathine (equivalent to 2 g. sulphamezathine) taken orally in about 250 ml. water containing one equiv. NaOH.)

Blood analysis				Urine analysis						
Time after dosing		Sulphamezathine concentrations (mg./100 ml.)		Time after dosing		Volume excreted	Sulphamezathine concentrations (mg./100 ml.)		Total recovered sulphamezathine (mg.)	
(hr.)	(min.)	Free	Total	(hr.)	(min.)	(ml.)	Free	Total	Free	Total
						Exp. 1				
0	25	0	11·2	1	50	284	1·04	193	2·9	550
1	30	0	8·2	4	5	271	2·15	220	8·7	1150
3	5	0	5·8	8	50	308	2·76	195	17·2	1750
5	15	—	3·4	12	35	279	1·47	75	21·3	1960
8	5	0	2·8	24	5	545	0·87	45·3	25·0	2211
—	—	—	—	27	55	330	0·45	6·4	26·5	2230
—	—	—	—	33	35	231	0·60	8·2	27·9	2250
						Exp. 2				
—	—	—	—	0	40	276	0·39	59·2	1·1	163
—	—	—	—	2	0	246	1·02	93·0	3·8	392
—	—	—	—	2	5	120	1·06	102	5·1	514
—	—	—	—	7	10	238	5·52	301	18·2	1231
—	—	—	—	13	10	330	3·53	102	28·4	1568
—	—	—	—	21	30	650	1·28	20·9	36·7	1704
—	—	—	—	25	25	930	0·40	2·97	40·4	1732

maximum within the first hour and then gradually fell off. The ratio concentration of free form/concentration of conjugated form decreased with time in blood and urine, indicating that the conjugation proceeded continuously. Virtually all the injected drug was recovered from the urine; the kidneys are thus the main organs of excretion. Over 80 % of the excreted drug was present in the conjugated form. On *oral* intake the maximum concentration in the blood was reached after 3–4 hr. and the proportion of the conjugated form again increased continuously from the start. Recovery from the urine was 97 % of the intake. Absorption from the intestine must therefore have been almost quantitative. Over 90 % of the excreted drug was conjugated (see also Volini, Engbring & Schorsch, 1945). In the urine the ratio free form/conjugated form fell continuously, and during the later stages of the excretion period 95–97 % of the excreted drug was conjugated. These data indicate that as long as free sulphamezathine is present, conjugation progresses. There is no suggestion of an 'equilibrium'

sulphamezathine ⇌ acetylsulphamezathine.

The reaction virtually goes to completion from left to right. This is borne out by experiments with acetylsulphamezathine. After oral administration of this substance no free sulphamezathine was detected in the blood, and only traces, hardly exceeding the limits of error, appeared in the urine (Table 3).

Sulphamezathine determinations on faeces after oral intake of 2 g. are shown in Table 4. The last three portions, which were collected 5 or more days after the oral intake and which may therefore be expected not to contain the drug, produced a colour equivalent to 2, 20 and 5 mg. total sulphamezathine respectively. These figures indicate the amounts of substances simulating sulphonamides. The first portion, which contained the non-absorbed drug, gave little more than the 'blank'. The analyses confirm the almost complete absorption of the drug.

Table 4. *Sulphamezathine in faeces after oral intake of 2 g.*

Period of collection of faeces after dosing (days)	'Sulphamezathine' found (mg.)	
	Free	Total
1–4	34	37
5–7	1·6	2
8–10	20	20
11–13	4	5

Pigeon. In the pigeon, in contrast to man, the reaction

sulphamezathine ⇌ acetylsulphamezathine

proceeds in both directions (Tables 5 and 6). When free sulphamezathine was given the ratio conjugated/free drug fluctuated around unity in the blood and in the excreta. After administration of the acetyl form, considerable amounts of the free drug appeared in the blood and in the excreta. At 4–6 hr. after the injection, about 50 % of the drug in the blood and excreta was free.

Rabbit. No significant amounts of free sulphamezathine appeared in the urine of rabbits after injection of acetylsulphamezathine (see Table 7).

Table 5. *Acetylation of sulphamezathine in the pigeon*

(Female pigeon 360 g. Intraperitoneal injection of 100 mg. sulphamezathine in 10 ml. 0·03 N-NaOH.)

Blood analysis					Analysis of excreta (urine plus faeces)						
Time after injection		Sulphamezathine concentrations (mg./100 ml.)		Ratio: Free/ Conjugated	Time after injection		Quantities of sulphamezathine excreted (mg.)		Ratio: Free/ Conjugated	Total recovered sulphamezathine (mg.)	
(hr.)	(min.)	Free	Total		(hr.)	(min.)	Free	Total		Free	Total
1	0	14·0	26·6	1·11	2	0	8·3	12·5	1·97	8·3	12·5
2	0	12·0	26·2	0·85	4	0	4·4	7·8	1·29	12·7	20·3
4	5	9·2	21·2	0·66	10	0	21·4	48·5	0·79	34·1	68·8
6	15	5·4	10·7	1·02	22	30	5·3	9·5	1·26	39·4	78·3
8	15	2·7	6·2	0·77	—	—	—	—	—	—	—
11	15	—	—	—	—	—	—	—	—	—	—

Table 6. *Deacetylation of acetylsulphamezathine in pigeons*

Blood analysis					Analysis of excreta (urine plus faeces)						
Time after injection		Sulphamezathine concentrations (mg./100 ml.)		Ratio: Free/ Conjugated	Time after injection		Quantities of sulphamezathine excreted (mg.)		Ratio: Free/ Conjugated	Total recovered sulphamezathine (mg.)	
(hr.)	(min.)	Free	Total		(hr.)	(min.)	Free	Total		Free	Total
Exp. 1. Female pigeon, 300 g. Intraperitoneal injection of 105 mg. acetylsulphamezathine (expressed as free) dissolved in 5 ml. 0·06 N-NaOH.											
—	—	—	—	—	1	50	3·4	47·2	0·078	3·4	47·2
—	—	—	—	—	3	50	6·0	17·2	0·54	9·4	64·4
—	—	—	—	—	5	40	4·7	10·7	0·78	14·1	75·1
—	—	—	—	—	7	15	4·0	6·2	1·82	18·1	81·3
—	—	—	—	—	23	30	2·4	2·9	4·8	20·5	84·2
—	—	—	—	—	32	40	—	2·2	—	—	—
—	—	—	—	—	73	—	—	7·3	—	—	—
Exp. 2. Same pigeon 15 days later. Intraperitoneal injection of 104 mg. acetylsulphamezathine (expressed as free) dissolved in 5 ml. 0·06 N-NaOH.											
0	40	2·9	17·1	0·204	7	30	2·1	34	0·066	2·1	32
1	35	4·4	18·9	0·304	24	0	5·8	13·8	0·73	7·9	40
3	35	5·1	14·2	0·56	72	0	—	0·2	—	—	40
5	5	3·3	4·5	1·50	—	—	—	—	—	—	—

Table 7. *Deacetylation of acetylsulphamezathine in the rabbit*

(Weight of animal 1250 g., 300 mg. acetylsulphamezathine (equivalent to 261 mg. sulphamezathine) injected subcutaneously as 10% solution of sodium salt.)

Urine analysis

Time after injection		Sulphamezathine excreted/sample (mg.)	
(hr.)	(min.)	Free	Total
5	35	1·29	230
8	15	0·52	12·9
24	20	0·80	16·3
27	5	Nil	1·59
47	20	Nil	3·71
55	30	Nil	1·66
73	30	Nil	0·102
	Total	2·6	266

Cat. The cat slowly hydrolyzes acetylsulphamezathine (Table 8). After a single oral dose of 150 mg., 11% of the recovered drug occurred free; after subcutaneous injection of 500 mg., 15% of the recovered drug was found to be free. The ratio free form/conjugated form rose steadily with time.

Dog. It has been reported that the dog cannot acetylate sulphonamides (Marshall, Emerson & Cutting, 1937). We find (Table 9) that on subcutaneous injection some conjugation occurred whilst the concentration of the drug in the body was relatively high. About 10% of the drug appearing in the urine was conjugated. As in the cat, injected acetylsulphamezathine was hydrolyzed (Table 10).

Enzymic hydrolysis of N^4-acylsulphonamides

N^4-Acetylsulphamezathine. Suspensions of sheep kidney were mixed with acetylsulphamezathine and phosphate buffer of pH 7·7 and kept at 38° C., octanol serving as a preservative. Unless stated otherwise, the suspension consisted of one part of minced tissue suspended in three parts of water. Samples were analyzed for free sulphamezathine

Table 8. *Deacetylation of acetylsulphamezathine in cats*

Urine analysis

Time after dosing (hr.)	Time after dosing (min.)	Sulphamezathine excreted/sample (mg.) Free	Total	Conjugated	Ratio: Free/Conjugated
Exp. 1. Weight of cat 1500 g.; 500 mg. acetylsulphamezathine, equivalent to 435 mg. sulphamezathine, injected subcutaneously as 10% solution of sodium salt.					
7	15	5·38	185	180	0·030
21	15	9·25	151	142	0·065
22	30	12·7	47	35	0·36
24	5	6·2	13·1	5·9	1·05
26	15	2·84	4·15	1·3	2·18
46	5	19·8	26·5	6·7	2·96
54	5	7·85	10·1	2·2	3·56
72	5	4·05	4·3	0·25	16
	Total	68	441	373	
Exp. 2. Weight of cat 1500 g.; 150 mg. acetylsulphamezathine equivalent to 130 mg. sulphamezathine, dissolved in 0·3 ml. 2N-NaOH plus 5 ml. water taken orally in milk.					
3	5	0·109	58·5	58·4	0·019
4	25	0·061	17·9	17·8	0·034
7	25	2·32	19·5	17·2	0·135
23	20	5·95	21·4	15·45	0·385
27	5	1·37	3·71	2·34	0·585
28	25	2·68	3·84	1·16	2·30
48	—	1·51	1·66	0·16	9·4
	Total	14	126·5	112·5	

Table 9. *Acetylation of sulphamezathine in the dog*

(Weight of dog 1190 g.; 100 mg. sulphamezathine injected subcutaneously as 3% solution of sodium salt.)

Urine analysis

Time after injection (hr.)	Time after injection (min.)	Sulphamezathine excreted/sample (mg.) Free	Total	Conjugated
3	20	13·7	16·8	3·1
6	20	16·3	19·2	2·9
22	10	31·3	32·5	1·2
28	—	5·1	4·5	—
30	—	1·9	1·4	—
46	—	1·54	1·54	—
	Total	69·8	75·9	7·2

Table 10. *Deacetylation of acetylsulphamezathine in the dog*

(Weight of dog 1190 g.; 100 mg. acetylsulphamezathine, equivalent to 87 mg. of sulphamezathine, injected subcutaneously as 3% solution of sodium salt.)

Urine analysis

Time after injection (hr.)	Time after injection (min.)	Sulphamezathine excreted/sample (mg.) Free	Total	Conjugated
4	—	1·28	17·8	16·5
7	—	1·97	24·6	22·6
24	10	15·6	31·2	15·6
37	20	0·78	1·23	0·45
48	—	3·3	2·6	—
	Total	22·9	77·4	55·2

after various intervals. Controls without tissue indicated that acetylsulphamezathine was stable under the experimental conditions. Table 11 shows that the hydrolysis was relatively slow and that the rate fell off with time. About two thirds of the added substrate was split after 9 days when higher concentrations of enzyme were used. Within the range of concentrations tested in this experiment the rate of hydrolysis was approximately proportional to the enzyme concentration, but in more concentrated kidney suspensions the rate did not increase with the tissue concentration (Table 12). The rate was approximately proportional to the substrate concentration (Table 13) when this was below 0·01M. The fall in substrate concentration may in part explain the falling off of the rate of hydrolysis with time. The inhibitory effect of high tissue concentrations was found in every tissue examined (kidney of dog, cat; liver of guinea-pig, sheep, cat, dog). The pH optimum of the enzyme in bicarbonate/CO_2 buffer was found to be between 7·3 and 7·5 (Table 14).

Among the tissues tested (Table 15) kidney cortex showed the highest, liver the second highest rate of activity. All the eight tissues showed some activity, though in some cases, notably brain and pancreas, the enzyme was very weak and ceased to act after a few hours. The enzyme was inactivated by heating the tissue suspension for 5 min. to 100°. Acetone powder of kidney cortex was inactive.

Other N^4-acetylsulphonamides. Table 16 shows comparative data of the rate of hydrolysis of six

different acetylsulphonamides. Acetyl-4:6-dimethoxysulphadiazine was much more rapidly hydrolyzed than acetylsulphamezathine, whilst hydrolysis of the other four compounds was slower than that of acetylsulphamezathine. Rose & Tuey (1946) noted a rapid hydrolysis of acetyl-4:6-dimethoxysulphadiazine *in vivo*. It has been stated that in the intact body sulphanilamide and sulphapyridine are more readily acetylated than sulphathiazole and sulphadiazine (Hawking, 1945). There appears to be no correlation between these differences and the rates of hydrolysis in sheep kidney extracts.

Table 11. *Hydrolysis of acetylsulphamezathine by sheep kidney*

(Each flask contained 4 ml. 0·2 M-Na_2HPO_4, 0·5 ml. 0·2 M-NaH_2PO_4, final concentration 0·002 M-Na acetylsulphamezathine, varying quantities of stock tissue suspension (minced sheep kidney cortex suspended in 2 vol. 0·01 N-$NaHCO_3$), and water to make up to 10 ml. 38°.)

Stock kidney suspension in 10 ml. sample (ml.)	Concentration of free sulphamezathine (mg./100 ml.) after			
	3·5 hr.	56 hr.	6 days	9 days
1·0	6·4	22·7	33·8	37·8
0·5	2·7	19·2	27·8	34·5
0·25	1·8	13·3	22·2	30·1
0·125	0·88	7·3	14·5	19·3
0·0625	0·60	4·0	8·3	12·8

Table 12. *Hydrolysis of acetylsulphamezathine at high concentrations of sheep kidney suspension*

(Conditions as in Table 11.)

Stock kidney suspension in 10 ml. sample (ml.)	Concentration of free sulphamezathine (mg./100 ml.) after	
	4 hr.	22 hr.
4	8·8	14·6
2	8·8	16·4
1	6·2	15·8

Table 13. *Effect of substrate concentration on the rate of hydrolysis of acetylsulphamezathine by minced sheep kidney cortex*

(Each flask contained 1 ml. stock tissue suspension; 4 ml. 0·2 M-Na_2HPO_4, 0·5 ml. 0·2 M-NaH_2PO_4, varying quantities of Na acetylsulphamezathine, total volume 10 ml.)

Concentration of acetylsulphamezathine (M)	Concentration of free sulphamezathine (mg./100 ml.) after	
	3·5 hr.	24 hr.
0·04	22·5	68·0
0·02	16·8	60·5
0·01	12·3	44·0
0·005	7·9	27·2
0·0025	4·4	11·8
0·00125	2·6	6·4
0·000625	1·1	2·5

Other N^4-acylsulphamezathines. The rates of enzymic hydrolysis of propionyl- and butyryl-sulphamezathines were roughly two thirds that of acetylsulphamezathine. The hydrolysis of benzoylsulphamezathine was extremely slow (Table 17).

Conjugation of sulphonamides in isolated tissues

Conjugation in different species. The experiments were carried out on sliced or minced material suspended in phosphate saline or bicarbonate saline. When mince was used the ratio of tissue to medium was between 1 : 5 and 1 : 10. The suspensions were shaken in Warburg vessels at 40°. The gas phase of the vessels contained O_2 or 5 % CO_2 in O_2.

One of us (Sykes, 1944), and Lipmann (1945) have reported independently that among the tissues of various laboratory animals pigeon liver is best suited for the investigation of conjugation of sulphonamides *in vitro* because this is the only tissue where the degree of conjugation *in vitro* was found to be of the same order as in the intact animal. Tissues

Table 14. *Effect of pH on the hydrolysis of acetylsulphamezathine*

(Na_2CO_3/$NaHCO_3$/CO_2 buffer; pH calculated from $pK_1 = 6·10$; $pK_2 = 9·79$ (see also Delory & King, 1945). In order to have containers which could conveniently be filled with gas mixtures from cylinders, Warburg vessels were used. They contained 0·5 ml. buffer; 3 ml. 0·005 M-Na acetylsulphamezathine and 0·5 ml. sheep kidney cortex suspension (1 part minced kidney, 5 parts water). Incubation 3 hr., 38°.)

Buffer mixture (made up to 0·5 ml. with N-NaCl)			Initial pH (calculated)	Free sulphamezathine found (mg./100 ml.)
M-Na_2CO_3 (ml.)	M-$NaHCO_3$ (ml.)	Gas mixtures		
0·25	0·25	Air	9·79	0·32
0·10	0·40	Air	9·20	0·63
0·05	0·45	Air	8·85	0·91
—	0·5	5% CO_2 in O_2	8·14	1·83
—	0·25	5% CO_2 in O_2	7·84	3·15
—	0·125	5% CO_2 in O_2	7·54	3·75
—	0·0625	5% CO_2 in O_2	7·34	4·20
—	0·5	100% CO_2	6·84	1·37
—	0·25	100% CO_2	6·54	1·26
—	0·125	100% CO_2	6·24	1·17

examined by us include the livers and kidneys of rat, guinea-pig, rabbit, cat, dog and sheep. Conjugation never occurred in kidney. In liver it was irregular, varying between 0 and 20 % of the added drug. In accordance with the findings of Klein & Harris (1938) rabbit liver was somewhat more effective than the livers of the other species, but yields were always small and irregular. There were no consistent effects with glucose, acetate, pyruvate, lactate, fumarate or tryptic casein digest. In view of the essentially negative nature of the results these experiments are not reported in full. The following experiments were all carried out on pigeons.

Table 15. *Hydrolysis of acetylsulphamezathine by various tissues of the sheep*

(Each test tube contained 4 ml. 0·2M-Na_2HPO_4; 0·4 ml. 0·2M-NaH_2PO_4; 2·5 ml. 0·04M-Na acetylsulphamezathine; 1 ml. tissue suspension; 2·1 ml. water.)

Tissue	Concentration of free sulphamezathine (mg./100 ml.) after 28 hr.	52 hr.	122 hr.
Kidney cortex	32	52	65
Lung	6·5	8·8	10·2
Liver	10·6	16·2	29·2
Brain	3·0	3·4	3·9
Pancreas	2·0	2·0	2·06
Spleen	8·8	9·7	11·0
Mucosa of jejunum	11·4	17·5	27·5
Blood (citrated)	7·0	9·2	11·1

No experiments appear to have been reported in the literature on the nature of sulphonamide 'conjugation' in pigeons. Experiments on the intact pigeon showed that the conjugation of sulphamezathine is essentially an acetylation. Three pigeons received subcutaneously a dose of 200 mg. sulphamezathine, as sodium salt, on three consecutive days and the excreta were collected. The injection caused the animals to empty their crops within about 30 min. and most of the subsequent food was also vomited during the 3-day period. The collected excreta were dried with anhydrous Na_2SO_4 and the dry homogeneous powder was extracted with acetone for 3 hr. in a Soxhlet. The extract contained about 200 mg. each of free and conjugated sulphonamide. The acetone was evaporated and the residue was ground up in a mortar with 2 ml. ice-cold 5 % H_2SO_4 to dissolve the free sulphonamide. The insoluble residue was washed twice on the centrifuge with ice-cold water and then dissolved in hot water. On cooling, 54 mg. acetylsulphamezathine crystallized (m.p. 237°). This relatively low yield was mainly due to hydrolysis of the N^4-acetyl group under the conditions of purification. Acetylsulphamezathine appears to be more readily hydrolyzed in faecal extracts than in pure solutions. After recrystallization from methanol a pure product was obtained which was identified by m.p. (243°), mixed m.p. and elementary analysis.

Time curves of acetylation and deacetylation in pigeon liver. Table 18 shows an example of the time curve of the conjugation of sulphamezathine in suspensions of minced pigeon liver. Conjugation proceeded steadily until about 55 % of the added drug had reacted; the reaction then came to a standstill, and under the conditions of the experiment this occurred after about 40 min. Similar degrees of conjugation were obtained irrespective of the initial concentration of sulphamezathine. The standstill was not due to the conjugating system, for addition of further substrate caused again conjugation of about 55 % of the added drug. This level is similar to that reached in the intact pigeon.

Table 16. *Hydrolysis of various acetylsulphonamides*

(Each test tube contained 4 ml. 0·2M-Na_2HPO_4; 0·4 ml. 0·2M-NaH_2PO_4; 1 ml. kidney cortex suspension; 2·5 ml. 0·02M-Na acetylsulphonamide; water to 10 ml.)

Substrate	Concentration of free sulphonamide (mg./100 ml.) after 4 hr.	29 hr.	117 hr.	Concentration of free sulphonamide calculated for complete hydrolysis (mg./100 ml.)
Acetylsulphanilamide	0·27	3·8	10·0	88·9
Acetylsulphamezathine	3·78	23·8	45·4	139
Acetylsulphapyridine	1·63	11·4	26·9	125
Acetylsulphathiazole	0·67	5·06	16·5	128
Acetylsulphadiazine	0·73	3·23	7·52	125
Acetyl-4:6-dimethoxysulphadiazine	14·3	66·2	123	147

Table 17. *Hydrolysis of various N^4-acylsulphamezathines*

(Conditions as stated in Table 16.)

Substrate	Concentration of free sulphamezathine (mg./100 ml.) after 21 hr.	46 hr.
N^4-Acetylsulphamezathine	11·3	17·3
N^4-Propionylsulphamezathine	6·8	10·8
N^4-Butyrylsulphamezathine	8·5	11·6
N^4-Benzoylsulphamezathine	1·6	1·8

Table 18. *Conjugation of sulphamezathine in minced pigeon liver*

(Minced pigeon liver suspended in 10 parts phosphate saline containing 0·001 M-sulphamezathine (27·8 mg./100 ml.) shaken in Warburg cups at 40°. Gas phase O_2.)

Time (min.)	Concentration of sulphamezathine (mg./100 ml.) Free	Total	Conjugation (%)
0	27·8	27·8	0
20	21·4	28·1	23
40	12·1	28·2	57
60	12·4	28·1	56
100	12·6	27·3	54

Table 19 gives the rate of deacetylation of sulphamezathine in pigeon liver on the same specimen under the same conditions. Deacetylation was considerably slower than acetylation. After 100 min. 27 % had undergone hydrolysis.

Table 19. *Deacetylation of sulphamezathine in minced pigeon liver*

(Conditions as in Table 16 except that 0·001 M-acetylsulphamezathine was the substrate.)

Time (min.)	Concentration of free sulphamezathine (mg./100 ml.)	Hydrolysis (%)
20	4·7	17
40	5·6	20
60	6·4	23
100	7·6	27

Effects of added substrates. Under the conditions of our experiments (see Table 18) acetate, pyruvate and acetoacetate had no effects on the rate and degree of conjugation, in contrast to the findings of Lipmann (1945). However, in view of the different experimental conditions, our results are not contradictory to Lipmann's. The tracer work of Bloch & Rittenberg (1945) together with the *in vitro* experiments of Lipmann (1945) leave little doubt that acetate can serve as a source for the acetyl group of acetylsulphonamides.

DISCUSSION

Acetylation in different species. It has often been stated that the dog is not capable of conjugating sulphonamides (Marshall, Emerson & Cutting, 1937). The data reported in this paper indicate that the differences between the dog and other species are quantitative rather than qualitative. All species examined can conjugate sulphonamides as well as hydrolyze the conjugated forms. According to the available data the rate of hydrolysis in extracts of minced tissues is of the same order of magnitude in different species. The degree of acetylation observed in the intact animal appears to be the resultant of the activities of the acetylating and deacetylating systems. For reasons so far obscure the deacylating system although present and active in tissue extracts of all species tested seems to be non-operative in the intact body in some species (man, rabbit) but active in others (dog, cat, pigeon).

'Equilibrium' between conjugated and free sulphonamide. The data given in Tables 2, 3, 5, 6, 7, 8, 9 and 10 indicate that after a single dose of acetylsulphamezathine the 'equilibrium' or steady state in the system:

$$\text{acetylsulphonamide} + H_2O \rightleftharpoons \text{sulphonamide} + \text{acetic acid}$$

appears to be attained in man and rabbit when virtually all the drug is present in the conjugated form, whilst in cat and dog it is reached when virtually all the drug is free. In the pigeon the steady state is reached when the free and acetylated forms are approximately equal in amount. It is very unlikely that these 'equilibria' are true thermodynamic equilibria, though the possibility cannot be excluded. In aqueous solution the thermodynamic equilibrium is attained when almost all the sulphonamide is free. By reducing the concentration of water the thermodynamic equilibrium would shift in favour of conjugation and it is possible that the enzyme is located in a non-aqueous phase of the tissue.

It must be pointed out that the quantitative data in the rates of acetylation and deacetylation in isolated pigeon liver (Tables 18 and 19) are not in full accordance with the conception that the 'equilibrium' is the resultant of the activities of two different enzyme systems. The rate of hydrolysis appears to be too slow to account for an 'equilibrium' at about 55 % acetylation. However, the activity of the deacetylating systems depends on factors which have not yet been studied in detail (such as the inhibitor present in high concentrations of tissue, see Table 12) and the discrepancy in the rates therefore does not necessarily invalidate the above conception.

Previous observations on the hydrolysis of acylaniline derivatives. Kohl & Flynn (1940) were the first to demonstrate the deacetylation of N^4-acylsulphonamides in tissue extracts. They reported that rat liver hydrolyzes N^4-acetyl-, -butyryl-, -valeryl-, -caproyl- and -heptanoylsulphanilamides; the rate increased with the chain length of the acyl group (which according to Table 17 does not apply to acylsulphamezathines). There are several observations recorded in the literature indicating that the hydrolysis occurs in the intact body. Ockerblad & Carlson (1939) found that blood and urine of dogs and human subjects contained free sulphanilamide after oral intake of N^4-acetylsulphanilamide. Rose & Tuey (1946) found 4:6-dimethoxysulphadiazine

in the blood of mice after injection of its acetyl derivative and concluded that the compound is rapidly deacetylated in the body. Michel, Bernheim & Bernheim (1937) showed that acetanilide is hydrolyzed by rat-liver and rat-kidney extracts and Elson, Goulden & Warren (1946) reported that acetanilide and acetylphenetidine ('phenacetine') are deacetylated by the intact rat.

Classification of the enzyme hydrolyzing N^4-acetylated sulphonamide. The enzyme splitting acylsulphonamides and related compounds belongs to the 'acylases', a term used by Abderhalden & von Ehrenwall (1931) to designate enzymes which hydrolyze substituted amides according to the reaction

$$R.\mathrm{CONH}R' \xrightarrow{+\mathrm{H_2O}} R.\mathrm{COOH} + \mathrm{NH_2}.R'.$$

Acylases are related to peptidases, differing from them in that the acyl residue of the substrate does not contain an amino group. Examples of acylases are hippuricase (where $R = \mathrm{C_6H_5}$ and $R' = \mathrm{CH_2}.\mathrm{CO_2H}$) and the enzymes described by Kimura (1929) which hydrolyze the *N*-benzyl, acetyl and formyl derivatives of α-amino acids. The substrates used in this paper are all acyl derivatives of substituted anilines. It remains to be investigated whether the enzyme described in this paper is identical with other acylases.

SUMMARY

1. Sulphamezathine and N^4-acetylsulphamezathine were given orally or parenterally to human subjects, rabbits, dogs, cats and pigeons, and the amounts of free and conjugated drug were determined in the blood and in the excreta. In man and rabbit conjugation appears to proceed as long as free sulphamezathine is present and the acetyl compound is not hydrolyzed in significant quantities. In contrast acetylation can be reversed in the pigeon, dog and cat. In these species injected acetylsulphamezathine is hydrolyzed. In pigeons there appears to be an 'equilibrium', or steady state, between free and acetylated sulphamezathine when about 55% of the drug is conjugated. In the dog acetylation only takes place when the concentration of the free drug in the body is relatively high and in the steady state the bulk of sulphamezathine is free.

2. Suspensions of minced mammalian tissues contain an 'acylase' first described by Kohl & Flynn (1940) which hydrolyzes N^4-acylsulphonamides. Among sheep tissues, kidney cortex, liver and intestinal mucosa show the highest activity. Some properties of the enzyme are described. The pH optimum is at pH 7·3. The highest rate of hydrolysis of six sulphonamides was given by acetyl-4:6-dimethoxysulphadiazine (Table 16). N^4-Acetylsulphamezathine was hydrolyzed more rapidly than the analogous propionyl, butyryl and benzoyl compounds (Table 17).

3. In sliced or minced liver or kidney of the rat, guinea-pig, rabbit, cat, dog and sheep conjugation of sulphonamides was found to be small and erratic. Minced, sliced or homogenized pigeon liver on the other hand readily conjugated sulphamezathine. The conjugation stopped when about 55% of the added drug was acetylated. The rate and degree of acetylation were not affected by acetate, pyruvate or acetoacetate. Acetylsulphamezathine was deacetylated in pigeon liver.

4. It is suggested that the position of the steady state in the system: sulphonamide ⇌ acetylsulphonamide is the resultant of the relative activities of the acetylating and deacetylating system. Though present in all species examined the deacetylating system appears to be inactive in the intact body of some species (man, rabbit). Discrepancies between the acetylating power of sliced or minced tissues and the intact body in rat, guinea-pig and rabbit may be connected with changes in the relative activities of the two enzymes, brought about by slicing and mincing.

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The Mechanism of Damage to the Bone Marrow in Systemic Poisoning with Mustard Gas

By DOROTHY MOYLE NEEDHAM, J. A. COHEN and A. M. BARRETT
Biochemical and Pathological Laboratories, Cambridge

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One of the most important features in recent investigations on mustard gas ($\beta\beta'$-dichlorodiethyl sulphide, referred to as *H* throughout the paper) is the focusing of attention on the systemic effects of this poison. The general appreciation in this country of the importance of this type of effect was caused by a series of reports by Cameron and his collaborators (Cameron, 1941*a*, *b*; Cameron, Short & Foss, 1942; Courtice & Cameron, 1942) in which, besides giving a summary of earlier observations, they described a number of pathological experiments of their own from which it becomes clear that *H* when applied in small doses to the skin of rats (5–10 mg.) or rabbits (10–15 mg.) causes, apart from its local effects, definite widespread changes throughout the organism. These changes are very severe in fatal cases, and they are here undoubtedly the only reason for death, because local symptoms are in these experiments either insignificant or (where the *H* is given by injection) non-existent.

According to Cameron, changes in the bone marrow are amongst the most specific and striking effects of this systemic poisoning. Here the white cell system is mainly affected: there is a degeneration of polynuclear leucocytes and myelocytes leading to a total disappearance of these cells; the bone marrow is fatty and oedematous; the megakaryocytes show dispersion and pyknosis of their nuclei; they are vacuolar and swollen and their boundaries are frayed out. Alterations in the red cell system are absent or much less marked and they usually occur at a much later stage. Leucopenia after an initial leucocytosis is probably mainly a result of these changes. The blood leucocytes also show marked degenerative changes. Among other systemic effects are an acute gastritis and changes in lymphoid tissue. The latter consist in depletion of lymphocytes, and in degeneration and focal necrosis in the spleen, lymph glands and lymphoid tissue.

The following experiments were undertaken in order to throw light on the biochemical mechanism underlying the systemic effects. In view of the indication from previous work on local lesions that enzymes connected with carbohydrate metabolism (phosphokinases) are especially affected by *H* (Dixon & Needham, 1946), the investigation of the glucose utilization of suitably chosen organs seemed most promising. We therefore studied the anerobic glycolysis of the bone marrow of poisoned animals; and in order to assess the specificity of the bone marrow damage we made similar observations in the spleen and brain. The damaged tissues were also examined histologically, and in a few observations in the early stages of poisoning we attempted to discover whether the histological or the metabolic changes could first be recognized. We were interested in this time relationship because, if there had been a significant interval between the first appearances of these two types of change, it might have thrown light on the pathogenesis of the lesions.

It seems highly probable that the widely distributed systemic changes are caused by some toxic substance, circulating most probably in the blood stream. We therefore tried to examine the properties of this compound by (1) testing the effect of serum from *H*-poisoned animals on the metabolism of normal marrow *in vitro*; (2) by comparing *in vitro* the effect of *H* on metabolism of marrow with that of *H* previously hydrolyzed in serum; (3) by observing the effects of excluding from the circulation part of the animal's marrow during the first period (up to 1 hr.) after injection with *H*; (4) by testing the possibility of the temporary storage of unchanged *H* in fat, and its subsequent release.

EXPERIMENTAL METHODS

Preparation of rabbit marrow for manometric experiments. The rabbit was stunned by a blow on the head and killed by bleeding. Immediately after death the femora and tibiae were dissected out, and both ends were cut off. In some cases the humeri were also used. The marrow was forced out of the bone by a blast of air into Ringer solution, and was then squeezed through coarse muslin and shaken. On standing, the fatty substance quickly rose to the surface and was pipetted off. The marrow cells were then spun down and resuspended in Ringer solution for the manometric experiments. By using a suspension of cells thus freed from most of the fat, it was possible to obtain quite uniform samples; the cells from the marrow of one animal were usually made up to about 8 ml. Duplicates of 0·5 ml. were used for the estimations of N content, and 2 or 3 ml. samples for the experiment. About 30 mg. dry wt./pot was a convenient amount for the glycolysis experiments; larger amounts were used for respiration measurements. When the manometric experiment was to be done in serum, the Ringer suspension, after removal of the samples for nitrogen estimations, was recentrifuged, and the cells were

made up to the same volume with serum. The Ringer solution had the following composition: 0·8% NaCl, 116 ml.; 1·0% KCl, 3 ml.; 1·12% $CaCl_2$, 2 ml. In the bicarbonate-Ringer, 20 ml. of the NaCl solution were replaced by 20 ml. of 1·26% $NaHCO_3$ solution.

Preparation of spleen. Since the preparation of satisfactory tissue slices from the spleen is difficult, a suspension of spleen cells was used. This was obtained by cutting the spleen into small pieces suspended in Ringer solution, and squeezing them through coarse muslin. The suspension was centrifuged, and further treatment was just as for the marrow.

Preparation of serum. Fresh rabbit blood was collected and defibrinated at once by shaking with glass beads. After straining through glass wool, it was centrifuged and the serum was pipetted off. It was then ready for use in the glycolytic experiments, but for respiration experiments it was necessary to remove bicarbonate and to prepare 'neutralized' serum, as described by Warren (1934). This was done by adding 0·1 N-HCl till the pH had fallen to 6·6, evacuating till no more CO_2 was evolved, and adding 0·4 M-phosphate buffer, pH 7·4, to make a final phosphate concentration of M/150. The final pH should be 7·4; this was checked, and when occasionally necessary, was adjusted by addition of 0·1 N-NaOH.

For the glycolysis experiments done in serum it was necessary to take account of the retention of CO_2 by the serum. The simplest method is to calculate new constants for the Barcroft apparatus, in which the effect of retention is included. The retention curves of the sera, giving the relation between the CO_2 retained and the pH, are first obtained as described by Brekke & Dixon (1937). The new constants are then calculated by the following method, for which we are indebted to Dr Dixon:

Let an amount of acid be produced such that the manometric reading (h) increases by 1 mm.

Let B_1 be the bicarbonate content before the change in μl. (N.T.P.) in the amount of serum used; B_2 the bicarbonate content after the change; C_1 the initial CO_2 pressure in mm. manometric fluid; C_2 the final CO_2 pressure in mm. manometric fluid, and c the correction factor for the manometer, i.e.

$$\frac{\text{change of reading}}{\text{change of pressure in experimental vessel}}$$

(see Dixon, 1943). Under our conditions this could be taken as 1, without appreciable error.

Let K be the constant of the manometer for CO_2 when there is no retention and K^s be the desired constant taking account of retention.

Then $$B_1 - B_2 = K, \quad C_2 - C_1 = c.$$

Then the change of pH corresponding to an increase of 1 mm. in the reading is given by

$$\Delta\,\text{pH} = \log\,(B_1 - K)/B_1 + \log\,C_1/(c + C_1)$$

from the Henderson-Hasselbalch equation.

From the retention curve, which is almost linear, the retention R corresponding to ΔpH is determined. The new constant is given by $K^s = K + R$. The retention correction varies slightly with the reading, but under our conditions the error produced by taking K^s as constant does not exceed 4%.

Preparation of marrow fat. We wished to know the effect upon normal cell glycolysis of adding the marrow fat from poisoned animals. It was found that the fatty layer separated off as described above contains nitrogen and shows glycolytic activity. In order to free the fat from metabolizing cells, the fatty layer was mixed with an equal volume of distilled water, allowed to separate out again and removed; the process was repeated. After it had been pipetted off for the third time, the fatty layer was centrifuged, the fat removed and excess water drained away on filter paper. In this way, plasmolysis of cells should be obtained and any enzymically active cell-free extract should be washed away. The fat prepared in this way did indeed show very slight or no metabolic activity.

Estimation methods. Anaerobic glycolysis was estimated by measuring the CO_2 produced in serum or in bicarbonate-Ringer solution in an atmosphere of 95% N_2/5% CO_2, in the Barcroft apparatus. Respiration was measured in the Barcroft apparatus in 'neutralized' serum, in an atmosphere of oxygen, in presence of KOH papers; a few experiments were done in the Dixon-Keilin apparatus in bicarbonate-Ringer in an atmosphere of 95% O_2/5% CO_2. In all this work, glucose was present, unless otherwise stated, in the serum or bicarbonate-Ringer solution, in concentration of 0·2%. In the glycolysis experiments with serum, bicarbonate solution was used in the left-hand pot, but in the respiration experiments more satisfactory results were obtained if the neutralized serum was used on both sides of the manometer.

The results for both glycolysis and respiration are expressed in μl. CO_2 produced or O_2 absorbed/mg. fat-free dry wt./hr. ($Q_G^{N_2}$; Q_{O_2}). The fat-free dry weight was obtained by estimating N in measured portion of the suspension and multiplying by the factor 6·85 (Warren, 1934) for marrow, and 6·25 for spleen. The incineration was carried out in conc. H_2SO_4, with a mixture of SeO_2, K_2SO_4 and $CuSO_4$ as catalyst.

Poisoning of animals. In all experiments except those specially mentioned, the dose of H for rabbits was 5 mg./kg. In most of the work, the pure redistilled H was dissolved immediately before use in thiodiglycol, to obtain a solution containing 50 mg./ml. The required amount of this was injected into the ear vein. In a few later experiments the H was dissolved in triacetin.

Histological methods. Material was fixed in Zenker formol, and paraffin sections were stained by Ehrlich's haematoxylin and eosin method and by the method of Barrett (1944) which gives results similar to those of Maximow's haematoxylin-eosin-azure method. In the rat experiments, Leishman-stained smears as well as sections were used, and in preparation of the sections the bones were decalcified by Custer's (1933) method.

EXPERIMENTAL RESULTS

Marrow

Glycolysis of marrow. The anaerobic glycolysis of normal marrow was first determined both in bicarbonate-Ringer solution and in normal rabbit serum. The results agreed with those of Warren (1934, 1940) in showing greater activity in serum (Table 1). In the absence of glucose there was practically no glycolysis.

Table 1. *Anaerobic glycolysis of normal marrow*

	$Q_G^{N_2}$ of normal marrow
In bicarbonate-Ringer	6·0, 6·5, 6·0
In serum	9·0, 14·0, 10·0, 9·5, 10·6, 10·5, 9·0

The activity in serum of marrow from poisoned animals, killed at different times after injection of *H*, is shown in Table 2 (see also Fig. 1).

Table 2. *Anaerobic glycolysis of poisoned marrow*

Time after injection of *H* (hr.)	$Q_G^{N_2}$ of marrow	Degree of histological abnormality
0	10·3*	—
4·5	7·6	Slight
10·5	7·3	Moderate
12	3·9	Moderate
15·5	2·7, 3·4	Moderate
21	<3·0	(Not examined)
48	<1	Severe
72 (in bicarbonate)	<2	Moderately severe
18 (by skin application)	2·5	Moderate

* Mean value for normal animals.

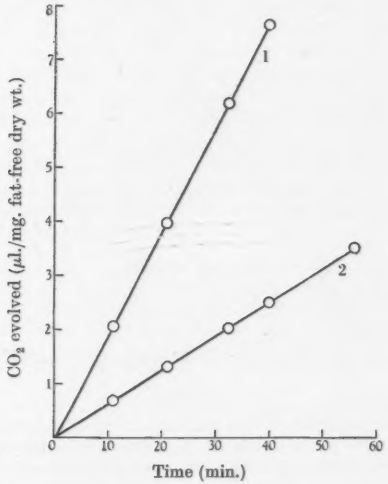


Fig. 1. Glycolysis of bone marrow from normal and *H*-poisoned animal in normal serum. 1, marrow from normal animal; 2, marrow from animal poisoned 12 hr. previously.

The progressive diminution in $Q_G^{N_2}$ with time after injection is seen; even as early as 4·5 hr. after injection, the value was slightly lower than any normal obtained, and by 12–15 hr. after injection the decrease is very marked.

One experiment was done to ascertain if similar metabolic changes accompany the histological damage in the bone marrow after application of *H* to the skin. *H* (55 mg. in 0·25 ml.) was painted on a shaved area of about 15 cm.^{2}. The results are given at the end of Table 2.

Histological evidence of damage was already present, though only slight, after 4–5 hr.; and it is interesting that it was the erythroblasts not the leucocytes which showed the most conspicuous abnormality at the early stage. The nucleus of the affected cells showed a kind of hydropic degeneration. While retaining its sharp outline and often several nodes of chromatin around its periphery, it was considerably swollen and completely empty-looking except for one or rarely two eosinophilic granules which seemed to be floating free in its interior. These granules probably represented nucleoli but were more strongly eosinophilic than the nucleoli of normal cells of the same kind. It was often difficult to decide whether cells affected in this way were erythroblasts or plasma cells: at first they were thought mostly to be plasma cells but it now seems certain that many if not all were nucleated red cells. A few myelocytes and other cells showed this hydropic nuclear degeneration. Granular leucocytes were still abundant but some showed karyorrhexis.

The histological changes seen in the later stages of damage for the most part agreed well with previous descriptions (Cameron, 1941*b*). In all except the very earliest stages, the cells of the granular leucocytes series were affected more than those of the erythrocyte series. The lack of parallelism between the hydropic nuclear degeneration and the other changes suggests that a different mechanism was involved. The most striking feature of marrows in which the damage is assessed in Table 2 as 'moderate' usually was a conspicuous decrease in the number of mature granular leucocytes, associated with an increase, apparently not merely relative, in the number of early myelocytes with scanty granules. These myelocytes were often unhealthy-looking and unusually large, but not rarely they were seen to be undergoing mitosis. Damage assessed as severe approached complete aplasia.

Respiration of marrow. The values found for respiration of normal marrow and those found after injection of *H* are given in Table 3. It will be seen that after about 14 hr., the respiration had fallen to only 30–50 % of the normal.

Table 3. *Respiration of marrow*

		Q_{O_2}
Normal marrow		2·0, 1·6, 1·2, 1·6, 0·9, 2·0
Poisoned marrow (hr. after injection)	15	0·45
	12·5	0·47
	14	0·84
	15	0·60

Effect of H and of H hydrolyzed in serum on marrow glycolysis in vitro. The suspension of marrow cells in Ringer solution was divided into three equal parts, which were centrifuged separately.

(*a*) The cells were suspended in $\frac{5}{2}$ ml. normal serum as usual; 0·025 ml. ethylene glycol monoethyl ether (e.g.e.) was tipped in on each side after equilibration, and the glycolysis was measured.

(*b*) The cells were suspended in 2 ml. normal serum, and after equilibration in the Barcroft pots, 0·025 ml. 4% *H* in e.g.e. was tipped into the suspension from a Keilin tube. (Bicarbonate, 2 mg. in 0·02 ml., had previously been added to this serum, the amount being equivalent to the acid expected from hydrolysis of the *H*.) An equal volume of e.g.e. was tipped into the left-hand pot. The glycolysis was measured.

(*c*) The cells were suspended in 2 ml. serum previously treated with *H* in the following way: 4 ml. serum (+4 mg. bicarbonate in 0·04 ml. water) were placed in the Barcroft apparatus in an atmosphere of 95% N_2+5% CO_2 at 38° and equilibrated as usual; 0·05 ml. 4% *H* in e.g.e. was tipped in and the reaction was allowed to continue till no more gas was evolved. This took about 45 min. The marrow cells were then suspended in 2 ml. of this serum and their glycolysis was measured.

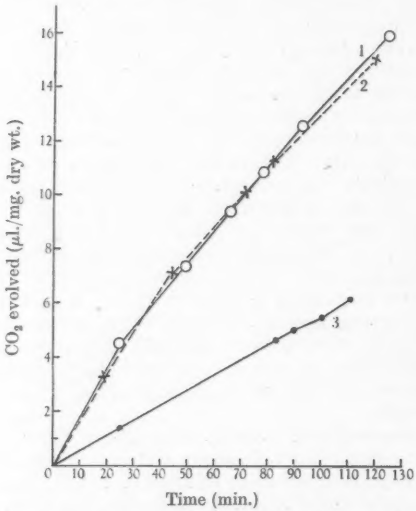


Fig. 2. Glycolysis of bone marrow under influence of *H* and of decomposed *H* in serum. 1, normal serum; 2, serum in which *H* has been decomposed; 3, serum containing *H*.

Fig. 2 shows the results. When *H* was added giving a concentration of 0·0026M, inhibition was immediate, and amounted to about 60%. After reaction of *H* in serum the same concentration had no effect.

The effect of serum from poisoned animals on the glycolysis of normal marrow. In these experiments the suspension of marrow cells in Ringer solution was divided into equal portions, which were centrifuged separately. The samples were made up with equal volumes of normal and of *H*-serum respectively. The poisoned sera used were: (*a*) obtained 4·5 and 21 hr. respectively after intravenous injection of the usual dose of 5 mg. of *H*/kg. (a separate rabbit being used for each experiment). There was no effect on the glycolysis of the cells suspended in them (see Fig. 3). (*b*) Obtained 2 and 1 hr. respectively after the injection of a very large dose (25 mg./kg.) a separate rabbit being used for each experiment. The rabbits were in both cases extremely ill, and would probably not have lived many minutes longer.

Fig. 3. Glycolysis of bone marrow in normal serum and in serum of poisoned animals. 1, serum of animal poisoned 4·5 hr. previously with *H*; 2, serum of normal animal.

In the first experiment with serum (*b*), obtained 2 hr. after the injection of *H*, an apparent 60% inhibition of glycolysis of marrow suspended in it was observed. It was found, however, that both bicarbonate content and pH of this serum were extremely low, the former equivalent only to 123 μl. CO_2/ml. of serum, and the latter 6·84. The glycolysis measurement was therefore repeated after addition of the calculated amount of sodium bicarbonate to bring the content back to normal.

In this medium the marrow glycolysis showed no significant deviation from normal ($Q_{O_2}^{N_2}$ in normal serum 10·5, in poisoned serum 9·0).

The experiment was repeated, using *H* serum 1 hr. after injection, the bicarbonate content having been adjusted as before. In this experiment, in order to give better opportunity for any toxic effect to become manifest, the marrow cells were suspended, before the glycolysis determination, in a comparatively large volume of serum, without glucose and in air. The freshly prepared marrow suspension in Ringer solution was divided into two parts and these were centrifuged. The cells were suspended in 8 ml. of normal and of *H* serum respectively and shaken at 38° in Barcroft pots, with the taps open for 80 min. The contents of the pots were then centrifuged, the cells were suspended in 1·25 ml. of the appropriate serum (now containing glucose) and the glycolysis was measured.

The $Q_{G}^{N_2}$ was 4·4 in the normal and 4·6 in the poisoned serum. (The low value for normal marrow is usual after much prolonged treatment.)

We have thus found no effect on the marrow glycolysis of serum from animals poisoned with *H*, apart from the effect due to low bicarbonate content of the serum after a very large dose.

The effect of suspending the marrow cells in the medium obtained by haemolyzing red blood cells from an animal 15·5 hr. after poisoning was also tested, and again the result was negative.

The effect of fat from marrow of poisoned animals on marrow glycolysis. It is known that *H* is more stable in a fatty than in an aqueous environment. If one could assume that *H*, after having been stored in the fat depots of the body is slowly released, the major difficulties in the explanation of the mechanism of the systemic action of *H* would be solved. The long latent period would become understandable and the postulation of a toxic intermediate would become unnecessary.

In order to examine the toxic properties of fat isolated from bone marrow of rabbits poisoned with *H* at different times previously we incubated for 45 min. 2 ml. of normal bone marrow suspension with 500 mg. of fat obtained from poisoned animals. A control was done each time with 2 ml. of the same normal marrow cell suspension and 500 mg. normal fat. After the incubation the anaerobic glycolysis was determined. For this purpose the contents of the Barcroft pots were made up to 3 ml. with bicarbonate solution containing glucose (to give the usual concentrations of bicarbonate and glucose). We usually allowed the glycolysis to go on for several hours in order to detect a possible quicker falling off at a late stage in the poisoned fat-normal marrow mixture as compared with the control. In all cases but one the glycolytic action of the fat involved was investigated, and found to be very low or nil. This glycolysis, when it occurs, is most probably to be attributed to cells or cell extracts which have escaped the washing.

From the results (Table 4), it is clear that the poisoned fat had no inhibitory influence on the glycolysis of normal bone marrow. It is possible that by the procedure of washing the *H* dissolved in the fat is removed. It seems likely, however, that if a considerable amount of *H* were really present in the fat before the washing, some would reveal itself afterwards during the long incubation with marrow (up to 3 hr.). Further, in Exp. 4, washing was restricted to a minimum. Here the marrow was collected dry (not into Ringer solution) and the fat was washed as rapidly as possible. The result was that fat so prepared kept some activity but inhibition did not occur.

Spleen

Glycolysis of spleen suspensions from normal and poisoned animals. These measurements were all done in bicarbonate (Table 5). After 21 hr., at a time when marrow glycolysis had fallen by about 70%, spleen glycolysis was unchanged. Even after 48 hr. it had only fallen by about 50%.

In the last experiment (48 hr.) histological examination showed considerable abnormality of the spleen. There was a conspicuous deficiency of lymphocytes in the Malpighian corpuscles, the absent lymphocytes being replaced by large pale cells with

Table 4. *Effect of fat from poisoned animals in marrow glycolysis*

Rabbit*			Total glycolysis in μl. CO_2					Glycolysis for marrow alone in μl. CO_2/flask/hr. after interaction with		
Wt. (kg.)	Killed after injection (hr.)	Fat ... Marrow...	Normal Normal	Poisoned Normal	Normal —	Poisoned —	— Normal	No fat	Normal fat	Poisoned fat
2·90	2·5		134	142	Considered negligible		—	—	134	142
2·35	5·0		110	104	0	0	—	—	110	104
2·45	13·0		—	258	Considered negligible		226	226	—	227
3·60	5·5		160	165	40	34·6	—	—	120	130·4†

Care was taken that in every experiment dealing with the effect of different fats on the same suspension of normal marrow cells, exactly the same amount of the latter was added to each flask, so that the rates of glycolysis obtained/flask could be compared directly.

* In each case 5 mg. *H*/kg. was injected intravenously.

† In this experiment the washing was restricted to a minimum.

oval nuclei, presumably reticulum cells. Polymorphonuclear leucocytes were fairly numerous but showed much evidence of destruction, karyorrhexis being common and free nuclear fragments abundant.

Table 5. *Glycolysis of spleen suspensions*

		$Q_G^{N_2}$
Normal spleen		5·5, 7·3, 5·8, 7·8
Poisoned spleen (hr. after injection)	10	5·5
	12	8·0
	15	6·5
	21	6·5
	48	3·5

In the experiment previously mentioned, in which *H* was painted on the skin instead of injected, and in which marrow glycolysis had fallen by about 75 %, the spleen glycolysis was almost normal, $Q_G^{N_2}$ 5·1. Histological examination in this case showed only moderate abnormality; there was some reduction in the number of lymphocytes in the Malpighian bodies, but polymorphonuclear leucocytes were abundant and mostly appeared healthy.

Respiration of spleen suspensions from normal and poisoned animals. The respiration of spleen suspensions in serum was also inhibited after *H* poisoning. The results (Table 6) suggest that in the spleen the inhibition of respiration may occur earlier than the inhibition of glycolysis.

Table 6. *Respiration of spleen suspensions*

		Q_{O_2}
Normal spleen		1·7, 1·9
Poisoned spleen (hr. after injection)	21	1·0
	24	0·7
	48	0

Brain

Anaerobic glycolysis and respiration of brain slices from normal and poisoned rats. Adult female rats, weighing between 180 and 240 g., were painted with a dose of 3 mg. *H* in 0·03 ml. e.g.e. on the shaved skin of the back. Since these experiments were more prolonged than most of those with rabbits, it was important that both control and experimental animals should receive the same amount of nourishment. As the *H*-painted animals scarcely eat, all the animals were given three subcutaneous injections daily of 3 ml. 50 % glucose. After 48 hr., the animals were killed by bleeding and slices were made from the cerebral cortex. Anaerobic glycolysis was measured in the usual way in bicarbonate-Ringer solution, respiration in the Barcroft apparatus in Ringer solution containing 0·0066 M-phosphate, pH 7·4, or in the Dixon-Keilin apparatus in bicarbonate-Ringer. The results for normal and poisoned animals are given in Table 7. The dry weight was obtained by removing the slices at the end of the experiment, washing them, drying at 110° and weighing.

It was found that, in sharp contrast to the marrow, after as much as 48 hr. from the time of poisoning, neither anaerobic glycolysis, nor respiration nor respiratory quotient was significantly altered.

The effect on bone marrow excluded from the circulation after injection of H

The simplest explanation for the activity of *H* would be that it is absorbed into the blood stream from the place of application, and carried unchanged with the blood to the bone marrow, which is poisoned as in the *in vitro* experiments previously described. The flaw in this conception is that it takes several hours for the intoxication of the marrow *in vivo* to make itself detectable by our methods, while *in vitro* there is a marked inhibition of glycolysis immediately after application of the poison. The reason for this difference might of course be the much higher degree of dilution in the experiments *in vivo*. In any case, one would expect with a mechanism of this kind (that is to say, with *H* acting *qualitate qua*) that the changes which condition the damage in the marrow should occur within a very limited period after injection because after that time all the free *H* will have disappeared in a blood medium, as will be shown presently.

This question was investigated by excluding a part of the marrow from the circulation for a certain

Table 7. *Anaerobic glycolysis and respiration of brain cortex slices*

		$Q_G^{N_2}$	
Normal brain	...	10·0, 16·0, 11·5, 12·0, 10·4, 10·0 (av. 11·6)	
Poisoned brain	...	7·0, 7·0, 13·0, 7·2, 9·8, 10·7 (av. 9·1)	
	Medium	Q_{O_2}	R.Q.
Normal brain	Phosphate-Ringer	−10·5	—
Poisoned brain	Phosphate-Ringer	−12·0	—
Normal brain	Phosphate-Ringer (no glucose)	− 4·5	—
Poisoned brain	Phosphate-Ringer (no glucose)	− 6·4	—
Normal brain	Bicarbonate-Ringer	−14·5	0·92
Normal brain	Bicarbonate-Ringer	−11·0	1·0
Normal brain	Bicarbonate-Ringer	−14·0	1·0
Poisoned brain	Bicarbonate-Ringer	−12·9	1·09
Poisoned brain	Bicarbonate-Ringer	−14·0	0·88

period after injection of the poison, to see if this would save this marrow in contrast to other parts of the marrow not excluded. The length of this period must depend on the rate of reaction of *H* in blood, and this was measured in the Barcroft apparatus at 38° by tipping a known amount of *H* into 3 ml. of blood after equilibration, and following the CO_2 output. The curves (Fig. 4) show that the reaction, as measured by the acid production, is about 60% complete in 20 min. and about 95% complete in 1 hr.

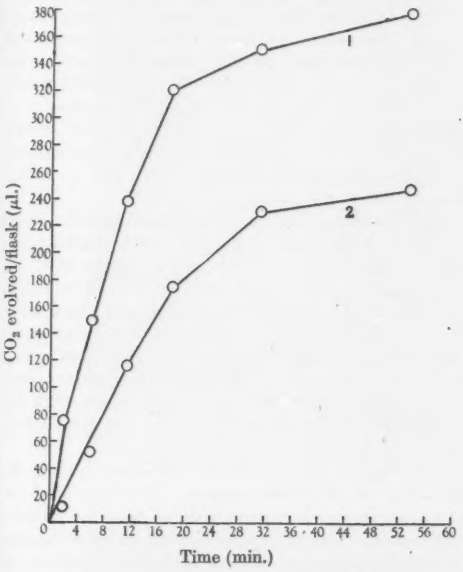


Fig. 4. Decomposition of *H* in heparinized plasma and whole blood. 1, *H* in plasma; 2, *H* in blood.

We used a technique similar to that described by Smith (1943) for the following experiments with rats. The abdomen was opened under ether anaesthesia and the vena cava and aorta abdominalis were clamped just below the origin of the renal vessels thus excluding the hind legs from the circulation. Then *H* in thiodiglycol was injected into the vena cava, centrally to the clamp, in a dose of 2 mg./kg. After removal of the clamp, the wound was closed. During the clamping the animal was kept warm, and the intestines were occasionally irrigated with saline at 37°. Controls were done with animals clamped for the same period, but only given the solvent. After the animals were killed, one humerus and one femur from each were examined histologically. The results are recorded in Table 8 and illustrated in Pl. 5.

In the control animals, the bone marrow in both the humerus and femur is either normal or showed a variable degree of acute inflammatory reaction presumably due to the operative treatment (see Pl. 5 C, D) but in the animals which received an injection of *H* a very different picture was seen (Pl. 5 A, B). The changes were similar to those produced by *H* in the bone marrow of rabbits, but it was more noticeable in the rats that eosinophil leucocytes were more resistant to the reaction of *H* than neutrophil leucocytes. In severely damaged marrows, when neutrophil leucocytes and indeed most other types of cell had almost completely disappeared, eosinophil leucocytes and tissue mast cells were still abundant. Despite the clamping of the abdominal aorta and vena cava for 20–60 min. after the injection of *H*, all the rats showed damage to the femoral bone marrow which was not appreciably less severe than that in the humerus. Thus the

Table 8. *Effect of exclusion of bone marrow from the circulation*

No.	Wt.	Duration of clamping (min.)	Injected with	Time elapsed before killing (hr.)	Histological damage	
					Humerus	Femur
1	168	20	TG	24	None	None
2	162	20	TG	24	None	None
3	194	20	—	24	None	None
4	209	20	2 mg. *H*/kg.	48	Severe	Severe
5	182	20	2 mg. *H*/kg.	24	Severe	Severe
6	167	20	2 mg. *H*/kg.	48	Severe	Severe
7	210	60	TG	24	None	None
8	208	60	TG	23	None	None
9	193	60	2 mg. *H*/kg.	21·5	Moderate	Moderate
10	247	60	2 mg. *H*/kg.	20	Moderate	Moderate
11	240	0	TG	72	None	None
12	233	60	TG	48	None	None
13	245	60	2 mg. *H*/kg.	48	Severe	Severe
14	182	60	2 mg. *H*/kg.	48	Severe	Severe
15	245	60	2 mg. *H*/kg.	72	Severe	Severe

TG = thiodiglycol. The *H* solutions were made up immediately before use, to contain 2 mg. *H*/ml. TG. The volume of the injection fluid used was in all cases 1 ml./kg. The rats used were all females.

poisoning of the marrow was not exclusively determined in the first hour after injection and therefore it seemed unlikely to be due to a direct action of unchanged *H* brought by the blood stream. However, the size of the dose of *H* may be important, because in another similar experiment, not recorded in Table 8, in which some of the *H* leaked from the syringe during the injection so that the effective dose was less than 2 mg./kg., the marrow in the humerus was severely damaged but that in the femur was little, if at all, affected.

These results agree with those obtained by Smith (1943) who, using small doses of mustard gas, found that bone marrow could be protected by exclusion from the circulation but using larger doses could confirm our results. Since the humerus was exposed to the action of any toxic substance in the blood both during the hour of clamping and afterwards, whereas the femur was only exposed afterwards, it is not surprising that with small doses the marrow of the humerus may be damaged while that of the femur is not. But with the dosage used by us, our experiments *in vitro* suggest that at least 95% of the *H* injected would have been hydrolyzed during the period of clamping; yet the marrow of the femur appeared as severely damaged as that of the humerus. We conclude that it is unlikely that the marrow damage was due to the direct action of unaltered *H*.

DISCUSSION

The inhibition of glycolysis in marrow, spleen and brain is not surprising, in view of what we know of the effect of *H* on the glycolytic enzymes *in vitro*, and on glycolysis in skin. The main aspects are: (*a*) the long delay, even greater than in skin, although *in vitro* the inhibitory effect was immediate; (*b*) the specificity of the effect on marrow which was much more affected than other organs in the early stages, both as regards cell damage and inhibition of glycolysis.

Concerning the mechanism of the delay, another significant finding, in the experiments involving clamping off of parts of the circulation, was that with the dosage which we employed the bloodstream appeared to be as efficient in intoxicating the tissue if it came in contact 1 hr. after the injection of *H* as immediately after injection. We have tried to test the two possible explanations for the lack of protection of the excluded marrow in the case of *H*:

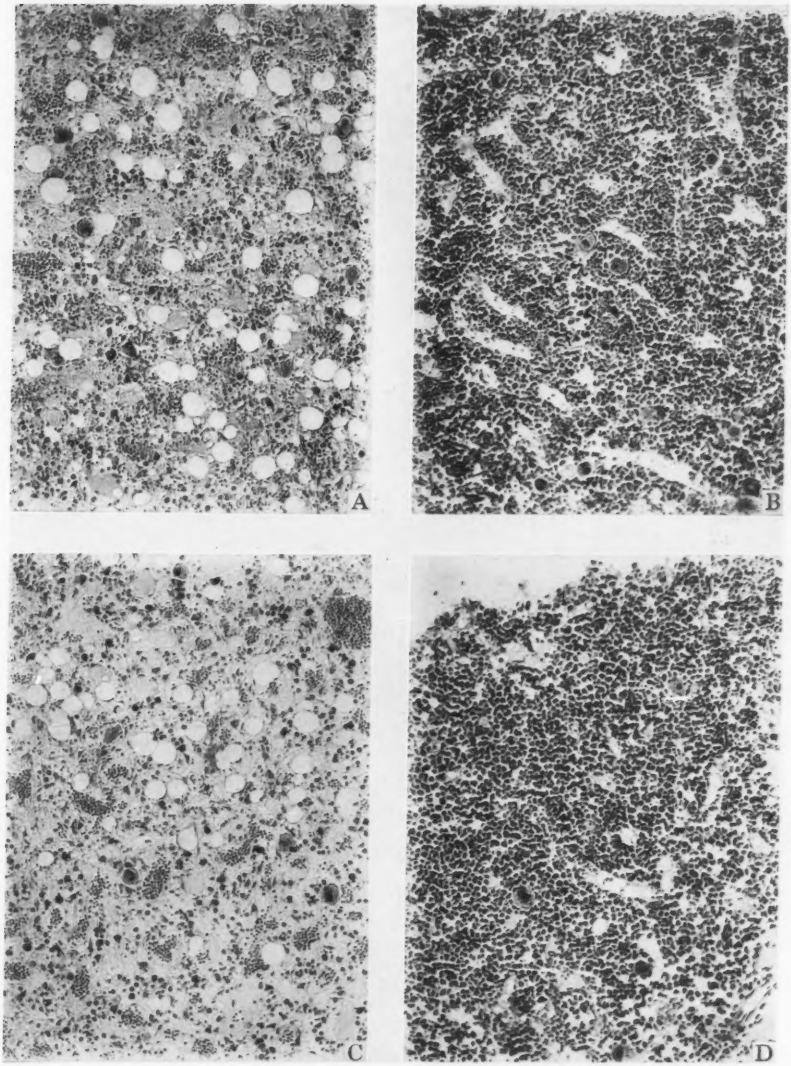
(*a*) The long continued circulation of a stable toxic product formed from *H*, which might for example, be a compound of *H* with protein. We obtained no inhibitory effect on normal marrow glycolysis by serum or haemolyzed red cells taken from animals after *H* poisoning. This cannot, however, be taken as ruling out the presence of an inhibitory substance in the bloodstream. In the experiments *in vitro*, the normal marrow cells can only be exposed to the *H* serum for a comparatively short time (3–4 hr.) and to a comparatively small volume. *In vivo*, the marrow is exposed for a much longer time before marked inhibition appears; and possibly as the blood circulates through the marrow, the toxic substance in it, even though present in low concentration, may react with some essential constituent in the marrow, so that finally the active amount of this constituent is reduced below some limiting value, and inhibition of glycolysis is manifest.

(*b*) The accumulation of unchanged *H* in fat, from which it is later slowly released into the aqueous phase. Here again, our negative results with fat prepared from poisoned marrow could not be taken as final, since it was possible, though not likely, that the fat was detoxicated during the preparation as carried out. Later experiments, however, using radioactive *H* showed that there is no specific accumulation of radioactive sulphur in bone marrow fat (Boursnell, Cohen, Dixon, Francis, Greville, Needham & Wormall, 1946).

The interpretation of the results showing inhibition by *H* of the marrow glycolysis and respiration is complicated by the fact that the relative amounts of white and red cells in the marrow change. The proportion of white cells becomes less with time after the poisoning and it is known from the work of Warren (1934) that the glycolytic activity of the white cells is greater than that of the red cells, while their respiratory activity is less. Clearly, a fall in the relative amount of white cells would (even in the absence of any direct effect of *H* on the cell metabolism) lead to a decrease in glycolysis and an increase in respiration, when these are expressed/mg. dry weight. What we have in fact found is a decrease in both glycolysis and respiration. The fall in respiration must be due to a direct effect upon the cells. We are inclined to think that the fall in glycolysis is also in part due to direct effect on the cells, especially the white cells, since it is marked at a time when the actual disappearance of leucocytes has not gone far, although the cells are abnormal. In the later stages, when the proportion of red cells is very high, the decrease in glycolysis is probably the result of two factors, the disappearance of the very active white cells, and the inhibition which may affect the red cells. In these late stages, the inhibition of respiration is probably chiefly due to the effect on the red cells.

SUMMARY

1. After intravenous injection of a lethal dose of mustard gas (*H*), the anaerobic glycolysis and the respiration of rabbit bone marrow were inhibited.



A, ×70, marrow from femur of rat killed 48 hr. after injection of *H* into the clamped inferior vena cava, the clamp being released 60 min. after the injection. Ehrlich's haematoxylin and eosin. B, ×70, control marrow from femur of normal rat. C, ×70, marrow from humerus of same rat as A. D, ×70, control marrow from humerus of normal rat.

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This inhibition was progressive, being just obvious after 4 hr. and very marked after 24 hr., and it ran parallel with the cell damage observed histologically.

2. Anaerobic glycolysis was also inhibited in the rabbit spleen, but only after a much longer time (48 hr.). At this late stage, histological damage was present.

3. No inhibition of anaerobic glycolysis or respiration was observed in brain slices from rats 48 hr. after poisoning.

4. We were not able to find any inhibitory effect on the anaerobic glycolysis of normal marrow cells by serum obtained from rabbits poisoned with the usual dose of *H*.

5. Neither haemolyzed red cells from a poisoned rabbit nor fat obtained from the marrow of such animals, had any effect on the anaerobic glycolysis of normal marrow cells.

6. Addition of *H* (0·0026M) to marrow cells *in vitro* caused immediate inhibition amounting to 50%. No effect was observed with the same amount of *H* after reaction with serum.

7. The acid production from *H* in serum and in whole blood took about 45 min. for completion at 38°; 85% was produced in 30 min.

8. Excluding part of the bone marrow of rats from the circulation for 1 hr. after injection of a lethal dose of *H* (2 mg./kg.) did not protect the marrow from the cell damage caused by *H*, as determined histologically.

This work was carried out for the Ministry of Supply in 1942–3, as part of the work of an Extramural Research Team under the direction of Dr M. Dixon, and was contained in a report to the Ministry dated 1944. We are indebted to the Chief Scientist Ministry of Supply for permission to publish this paper. We would also express our gratitude to Dr M. Dixon for his constant advice and interest.

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* Information available on application to Ministry of Supply, London.

Precipitation of Steroid Ketones with Digitonin

By G. A. D. HASLEWOOD, *Guy's Hospital Medical School, London, S.E.* 1

(*Received* 15 *April* 1947)

It is frequently held that a steroid is precipitable by digitonin by virtue of a free 3-hydroxyl group of the β-configuration (formula (I)). There are in fact at least four known exceptions to this rule, namely, *allo*-pregnan-3(β)-ol-20-one acetate, *allo*pregnane-3:20-dione (Butenandt & Mamoli, 1935), 'α'-oestradiol and its 3-benzoyl derivative (Wintersteiner, 1937). In the course of some work on the metabolism of cholest-4-en-3-one, it was discovered that this substance also is precipitable by digitonin. The following is a brief account of this reaction applied to some steroid ketones.

METHODS

The ketones, together with four parts by weight of digitonin, were dissolved with heating, in 90% ethanol-water. After at least 24 hr. at 18–20°, the digitonides were collected, washed with cold 90% ethanol-water, and ether, dried at *c.* 70° and decomposed by a single pyridine-ether treatment in the usual way. The ketones, recovered by evaporation of the washed ether, were crystallized from dilute ethanol. Table 1 shows the weights of digitonide and of crystalline recovered ketone. The amounts used were 50 mg. of ketone and 200 mg. of digitonin in all cases except that of progesterone, when 25 mg. of ketone and 100 mg. of digitonin were used. Recovered ketones were identified by mixed melting-point determinations.

The digitonin used in the above experiments did not itself crystallize out in 24 hr. at 18–20°, although it sometimes did so on longer standing. Unlike the precipitates of digitonides (except, perhaps, that from progesterone) the digitonin crystals themselves were readily soluble in cold water.

Table 1. *Yields of digitonides and of recovered ketones from digitonin precipitation in 90 % ethanol. Amounts of starting materials given in text*

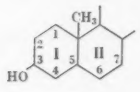
Steroid	Vol. of 90 % ethanol used to dissolve the mixture of steroid and digitonin (ml.)	Weight of digitonide (mg.)	Weight of recovered ketone (mg.)
Cholestanone	16	170	40
Coprostanone	5	0	0
Cholest-1-en-3-one	5	0	0
Cholest-4-en-3-one	5	190	40
Cholest-5-en-3-one	10	180	35
β-Sitost-4-en-3-one	5	180	40
Progesterone	2	60	10

RESULTS

These are shown in Table 1.

DISCUSSION

Since cholest-4-en-3-one (II) yields a digitonide whilst cholest-1-en-3-one (III) does not, it might seem that the reaction depended upon enolization from C_2 to give a 3(β)-hydroxyl group (IV); if this were so, it is difficult to see why coprostanone (V)



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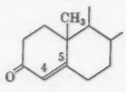

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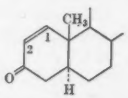

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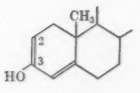

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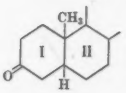
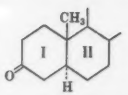


V



VI

does not react. The absence of the reaction with coprostanone (V) in contrast to cholestanone (VI) suggests that a *cis*-configuration of rings I and II is less favourable to digitonide formation than a *trans*- or flat (Δ^4- or Δ^5-) configuration. Nevertheless, the lack of precipitation with cholest-1-en-3-one (*trans*) remains unexplained.

From a consideration of the literature it is clear that digitonin precipitation does not depend only on a 3(β)-hydroxyl group, and may fail when such a group is present (cf. Butenandt & Fleischer, 1937; Reichstein & Gätzi, 1938; Spring & Swain, 1941; Wintersteiner & Ruigh, 1942). The reaction is evidently complex, but no steroid without an oxygen atom at C_3 has so far been found to precipitate with digitonin. Digitonin precipitation can be given approximately quantitative expression (Noller, 1939; Haslewood, 1948) and the figures obtained may assist in planning experiments and in interpretation of their results, especially where studies of steroid metabolism are concerned.

SUMMARY

Digitonides have been prepared from certain steroids with a keto group at C_3: the ketones have been recovered from these derivatives.

The author thanks the following for gifts of steroids: Dr C. L. Hewett (Organon Ltd.) for progesterone, Dr R. P. Jacobsen (Worcester Foundation, Mass., U.S.A.) for cholest-1-en-3-one and Dr E. R. H. Jones (Imperial College) for cholestanol, cholestanone and β-sitost-4-en-3-one.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The Biochemical Society and the Society for General Microbiology held a joint discussion meeting in the London School of Hygiene, Keppel Street, London, W.C. 1, on Saturday, 2 November 1946, *at* 11.15 *a.m. The subject of the discussion was 'Quantitative Biochemical Analysis by Microbiological Response' and the following communications were given:*

Introductory Survey. By B. C. J. G. Knight

Assay of essential metabolites. Why is it that micro-organisms can be used to assay a wide range of biologically important substances ranging from amino-acids and the vitamins of animal nutrition to certain groups of synthetic chemotherapeutic substances? The general answer appears to be that all cells use certain common metabolic processes in their basal cellular biochemistry, the sheer biosynthesis of living matter. This is the biochemical reflexion of evolutionary development. Suitable micro-organisms show biological responses to many of the compounds—essential metabolites—which play a part in these common metabolic processes.

Although these metabolic reactions are common to the widest variety of cell, the means by which cells procure the essential metabolites is different from organism to organism because of differences in synthetic ability. The essential metabolites, or parts of them, appear as essential nutrients for particular species or strains when these are unable to synthesize these substances. On this depends the choice of a suitable test organism and the chemical specificity of any particular assay. This common cellular biochemistry is the basis for the general applicability of microbiological assays to growth factors for any kind of organism. This relationship to a common biochemistry of cell metabolism imposes a limitation in that those physiological functions related to the functioning of multicellular organisms (e.g. the nervous and endocrine systems) do not find analogies in micro-organisms. Broadly speaking it is only the basal cellular biochemistry which is common to micro-organisms and all higher forms, and in general microbiological assays are not available for the substances involved in the physiological properties differentiated in higher organisms. Thus the vitamins A, C, D and E which are concerned in the maintenance of tissue structures characteristic of the more highly organized Metazoa are not in general assayed by microbiological methods.

The assay of amino-acids by methods involving microbiological growth responses is possible because bacteria exist which cannot synthesize certain essential amino-acids and thus show a response when the appropriate amino-acid is added to a suitable deficient medium.

Thus the first general type of assay method entails finding an organism which cannot synthesize a given essential metabolite or part of one, so that the organism will give some growth response proportional to the amount of that substance when it is supplied with it.

All types of micro-organisms have been or could be used, yeasts, bacteria, microfungi and protozoa, because of the unity of the biochemistry of cell metabolism in all forms of living matter.

In their practical aspect microbiological assay methods are valuable because of:

(i) the speed with which the test organisms react;

(ii) the small quantities of substances needed to obtain a specific and measurable response;

(iii) the relatively small scale of the experimental set-up and organization which permits relatively easy multiplication of the number of determinations.

Assay of anti-metabolite inhibitors. Methods of measuring and comparing the activity of synthetic growth inhibitors which have been modelled on the structures of essential metabolites, i.e. the class of anti-metabolite inhibitors exemplified by the sulphanilyl group of anti-bacterial drugs, are the obverse aspect of the assay methods for essential metabolites previously discussed.

Assay of natural antibiotics. At present, assay methods for this class of compound are direct and empirical, the test for activity being the power to inhibit growth of representative organisms for which inhibitory action is being sought. The existence of resistant strains has to be considered.

Assays using specific enzyme systems. In nature are to be found micro-organisms capable of performing almost every possible chemical reaction relating to biological material, many of which are not known to take place in animal or plant metabolism. These reactions are performed by enzyme systems operating under physiological conditions and often show high chemical specificity. It may be expected that search will reveal in some micro-organism or other an enzyme system to carry out almost any desired chemical reaction. Potentialities for the development of assay methods are very great. As examples of the deliberate search for such enzyme systems may be mentioned: (*a*) the selection of bacteria capable of decomposing, specifically, certain of the type specific capsular polysaccharides of pneumococci (Dubos & Avery, 1931); (*b*) an organism specifically attacking creatinine (Dubos & Miller, 1937; Miller & Dubos, 1937); (*c*) an organism specifically decomposing *p*-aminobenzoic acid (Mirick, 1943).

The general method used for the selection involved cultivating organisms from soil in suitable selective media containing the compound to be attacked, under conditions favouring selection and the development of adaptive enzymes capable of attacking the pure compound. Assay methods for creatinine and *p*-aminobenzoic acid were worked out which could be used under conditions where direct chemical methods were useless.

Enzymic antigens. The evaluation of many antisera depends on having available standard test doses of antigen derived from specific bacteria. Many branches of immunological assay can then be looked upon as examples of microbiological assay. Some immunological species or type specific antigens have enzymic properties. Thus the α-toxin of *Cl. welchii* is a lecithinase, splitting off phosphocholine (Macfarlane & Knight, 1941); the *Kappa* toxin of *Cl. welchii* is a collagenase (Oakley, Warrack & van Heyningen, 1946); the β-lecithinase of *Cl. oedematiens* type B and the γ-lecithinase of *Cl. oedematiens* type A, although probably identical in the biochemical reaction they bring about, are immunologically distinct (Oakley, Warrack & Clarke, 1946).

With these enzymic antigens assays of the corresponding antisera can be carried out by methods involving the specific inhibition of the corresponding enzyme by the antiserum, and the antigens themselves can be measured in terms of the specific chemical reactions they bring about.

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Some Principles in the Use of Microbiological Methods. The Types of Response Measured and their Relationship to Substances being Determined. By HENRY MCILWAIN

This account is classified according to the complexity of the system involved in giving the response, which is measured, to the added substance which is under investigation. It proceeds from methods in which such connexion is most immediate, to those of less directness.

Methods I, employing non-proliferating organisms or enzyme preparations from them. (*a*) Dr Gale's (subsequent) account exemplifies well those instances in which the substance being determined acts as substrate for an enzyme preparation, while one of the products (e.g. CO_2) or another reactant (e.g. O_2) are measured. (*b*) In other methods the substance being determined acts as coenzyme and a product of the reaction catalyzed, or a coupled reaction (e.g. reduction of methylene blue) are measured. The methods are more sensitive than are the preceding class; thus 1 mol. of cozymase yields *c.* 3000 mol. CO_2/hr. with apozymase, glucose, hexose diphosphate and inorganic salts (Myrbäck, 1933; Axelrod & Elvehjem, 1939; Lennerstrand, 1941). With carboxylase from yeast the corresponding factor is 79,000 (Green, Herbert & Subramanyan, 1941).

Methods I (*a*) and (*b*) are the most rapid of microbiological assays, the reaction usually occupying 10–60 min. An advantage of microbiological over other sources of enzymes for such purposes lies in their controlled production, especially by deliberate selection or adaptation of the organisms from which they are derived. This may be illustrated by a further manner of using the reagents, (*c*) in which one component of a mixture is selectively destroyed. Thus by a preparation from a soil organism which inactivated nicotinic acid but not nicotinamide, small quantities of the two substances could be determined by assay before and after treatment (Allinson, 1943).

The specificity of response is greater in methods I than in others which are to be discussed, because of the relative simplicity of the biological system. But even a single enzyme is rarely completely specific. A microbial preparation usually contains many enzymes. One way in which these can interfere with assay is by causing synthesis or breakdown of the substance being determined. Thus apozymase responds to coenzyme II after a short time interval, during which it is converted to cozymase (Euler, Adler & Eriksen, 1937; Euler & Adler, 1938). This can be avoided by the use of inhibitors (Lennerstrand, 1941). A phosphatase of carboxylase preparations inactivates cocarboxylase (Westenbrink, van Dorp, Gruber & Veldman, 1940).

Methods II, measuring growth. The following are some stages in the relationship between added growth essentials and growth, whether this is measured by length, area, mass or by dependent properties such as light absorption. (*a*) Assimilation. This often involves chemical change, as of glutamic acid by streptococci (Gale & Taylor, 1946) or of cocarboxylase by yeasts. (*b*) Elaboration to functioning forms. (*c*) Reaction of the functioning forms as indicated above. (*d*) The coupling of (*c*) with growth. (*e*) Further changes, e.g. of inactivation, in the substance added or its derivatives.

It is necessary to follow the course of growth to understand the relationship between added substance and response given, though when growth-characteristics are known this is normally dispensed with, and the quantity of growth determined after a chosen interval. Growth may involve: (i) an initial slow or lag phase, (ii) rapid, usually logarithmic, phase(s), (iii) slower or stationary phase(s). Deficiencies in particular media constituents can affect particular phases in various ways (Dagley & Hinshelwood, 1938; Lodge & Hinshelwood, 1939; McIlwain, 1944). Growth may be limited in amount without alteration of other characters; lag may be increased, growth rate reduced, or various effects combined. The first situation is probably the easiest to adapt to assay as it allows a long period of stable readings and by empirical choice several current methods appear to use

growth of this character. It is relatively easy in methods at all promising to obtain regular standard curves for the pure substance being determined. Difficulties arise from other substances added with biological materials being assayed and the effects of these on the different phases of growth give data of value in choice of method.

Methods III, measuring metabolic products of organisms whose growth has been conditioned by the substances being assayed. Metabolic reactions so employed include acid formation (titrated or evaluated gasimetrically), O_2 uptake, or more specialized ones such as $NO_3^- \rightarrow NO_2^-$ (Hoagland & Ward, 1942). Factors affecting the relationship between added substance and measured response include all those previously discussed, and also others concerning the metabolic activities of the organisms after growth.

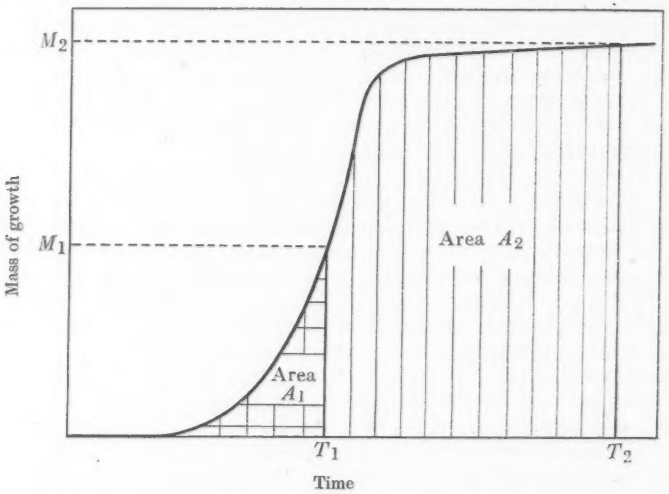

Fig. 1. Relationship between results obtained by methods II and III. At times T_1 and T_2, methods II give mass of growth M_1 and M_2, and methods III quantities of product dependent on the areas A_1 and A_2.

The relationship between these methods and methods II is, under certain conditions, as shown in Fig. 1. Results by methods III approximate to direct proportionality to those of methods II only if lag and logarithmic phases form a small proportion only of the total growth. Thus, even if mass of growth varies directly with quantity of added substance, acid formation may not do so; in practice, to approach proportionality, 'growth' is commonly allowed to proceed for longer times (up to 3 or 4 days) in methods III than in methods II. Variations in lag, e.g. of 3–4 hours, and in growth rate, which may not affect methods II will affect methods III; excess of all requirements in growth and fermentation (or other subsequent reaction) is especially necessary. If the metabolic activities measured are not a constant property of the test organism, but vary, for example, with age of culture, the quantity of secondary product will not be in proportion to the shaded area of Fig. 1. A special instance of this results from the inactivation of metabolites which frequently accompanies growth (see above; Morel, 1943; McIlwain, 1946).

No one group of the methods I–III is markedly more sensitive than others in determining a given substance. The main factors conditioning sensitivity appear to be the quantities of the particular substance which micro-organisms contain or with which they react, and which are a reflexion of the biochemical role of the substance concerned.

Biotin, pantothenate, or nicotinic acid occur in some mμmol./mg. dry weight of cells, and are determined in much smaller quantities by all methods than are the α-amino-acids of which some μmol. occur/mg. dry weight.

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Statistical Aspects of Microbiological Assays. By D. J. Finney

The biochemist need not study in detail the statistical theory which enters into the design and analysis of a good assay, but, if there is to be effective co-operation between him and a statistical adviser, he must have some knowledge of the principles guiding the statistician. The function of the statistician is not only to assess the validity and precision of assay results but also to recommend modifications in the design of an assay which, independently of changes in experimental technique, may be expected to improve the precision without increasing the labour; he cannot do this successfully unless he has some understanding of the biochemist's problems.

A microbiological assay is dependent upon the relationship between the *response*, a measurement of the growth of a micro-organism, and the *dose*, the amount of a standard preparation of the factor to be assayed which induces this response; the amount of the factor present in a test preparation is then estimated by comparing the responses produced by doses of this preparation with the standard relationship. If the dose-response curve for the standard preparation remained unchanged from day to day, it could be determined accurately once, and future assays made by reference to that determination. Experience shows this condition to be seldom satisfied, and therefore, in order to ensure comparable data, responses for both the standard and the test preparation must be measured on the occasion of each assay (Finney, 1947; Wood, 1946). Each response will be subject to the sources of variation generally grouped under the head of 'experimental errors', and the computation of the potency must make use of the response curves for the two preparations; in a valid assay, the two curves must be identical except for a multiplicatory factor in the measurement of the dose, which factor is the potency of the test preparation relative to the standard (Finney, 1945; Wood, 1945). Since responses for the two preparations enter symmetrically into the estimation of potency, the interests of precision are best served by measuring equal numbers of responses for each, rather than by expending more effort on the standard at the expense of the test or vice versa (Wood, 1946; Wood & Finney, 1946).

In many types of assay, over a considerable range of doses the response curve is a straight line. If the line for the standard preparation is

$$y=a+bx,$$

that for the test preparation must be

$$y=a+bRx,$$

where R is the potency which is to be estimated. Thus R is the ratio of the slopes of the two lines. If the size of an assay is limited by the total number of independent responses that can be produced and measured, the most precise estimate of R would be obtained by using only one dose of each preparation together with a blank or zero dose. In general, however, this common-zero three-point assay is unsuitable, as it assumes linearity of the response curves without permitting any test thereof. Considerably better is the common-zero five-point assay, using single and double doses of each preparation as well as the blank; the double dose of either should be the highest that is thought almost certain to be on the linear portion of the curve. If the number of responses to be measured is divided equally between the four doses and the blank, a satisfactory compromise is effected between the conflicting claims of precision in the estimate of potency and sensitivity in the test of linearity. For certain purposes, more complex schemes using several different dose levels may be advantageous, even though they involve a further small loss in the precision of R; there is seldom any reason for unequal division of the number between the various levels, or for using more levels of the standard than of the test preparation, though this last clause may need modification if several test preparations are to be assayed simultaneously (Bliss, 1946; Wood & Finney, 1946).

In types of assay for which the dose-response relationship is not linear, a transformation of the measurements of dose and response may simplify the computations. Often a straight line is obtained if the response is plotted against the logarithm of the dose; the lines for the two preparations must then be parallel and a good assay design should permit tests of both linearity and parallelism (Bliss & Marks, 1939*a*, *b*). A four-point assay design, using two doses of each preparation, is widely used in macro-biological techniques and also, for example, in the cylinder plate method of penicillin assay; it provides only for a test of parallelism, and should not be adopted unless there is a practical certainty of linearity. A design using three dose levels of each preparation instead of two, involving only a slight loss of precision in the estimation of potency, also gives data for a linearity test, and is preferable for many purposes. For more complex response curves, when no simple transformation is available, the labour of exact statistical analysis is likely to be prohibitive. A suggestion by Ipsen (1941) is capable of adaptation to this problem, but its practical utility can only be assessed after extensive trials. It would consist in a preliminary determination of a standard response curve which, though not remaining fixed from day to day, could in future be used as an empirical transformation for converting standard and test responses to 'equivalent doses'; these should be linearly related to the actual doses of the assay, and, with some modification, standard methods could then be applied to the transformed data.

When assays are carried out as routine checks on large-scale production, it is important to standardize the computations in a form requiring the minimum of arithmetical labour. Often purely graphical methods can replace arithmetical analysis, but precautions against unduly subjective judgements must be taken. A system of control charts may be useful as a means of keeping a continuous watch on the validity and precision of an assay technique (Knudsen, 1945; Knudsen & Randall, 1945).

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The Use of Enzyme Preparations from Bacteria for the Estimation of Amino-Acids.

By E. F. GALE

The first essential of a microbiological method of assay is that it should be specific for the substance to be estimated. Many enzymes are specific towards a single substrate and if such an enzyme can be separated from other similar enzymes and its action followed quantitatively, it can be used for the assay of its substrate. Attempts have been made in the past to utilize bacterial enzymes for the assay of amino-acids but preparations of deaminase systems have proved unsatisfactory since the partially purified preparations have contained traces of other ammonia-forming enzymes. The discovery of the amino-acid decarboxylases of bacteria has opened up a field of investigation which has so far provided enzyme preparations for the estimation of six amino-acids (Gale, 1945, 1946*a*).

When grown under acid conditions in the presence of the specific substrate certain bacteria produce amino-acid decarboxylases which attack their substrate according to the equation:

$$R.CHNH_2.COOH = R.CH_2NH_2 + CO_2.$$

Since the reaction proceeds quantitatively and at an acid pH, the CO_2 liberated can be measured manometrically and used to estimate the amount of the amino-acid substrate. Six decarboxylases have been identified and these are distributed in a haphazard fashion amongst bacterial species (Gale, 1946*b*). It has been possible to select organisms which can produce only one of these enzymes or, in other cases, to treat non-viable preparations in such a way that only one enzyme survives treatment. The methods of preparation and properties of the six amino-acid decarboxylases are summarized in Table 1. In each case a preparation is obtained which, when shaken with a mixture of amino-acids, will decarboxylate its specific substrate and so afford a means of estimating that amino-acid in the mixture.

Before such a method can be used as a routine method of assay it must satisfy certain conditions:

(1) *The preparation must attack the specific substrate only.* The action of the preparations has been tested against 22 amino-acids in pure state. Each preparation decarboxylates one only of these amino-acids. Substitution of the α-NH_2, the —COOH or the third terminal polar group renders the substrate immune to attack; consequently the preparations estimate the free amino-acids only. Substitution of hydroxyl-groups in the substrate molecule in a position other than in the three essential polar groups slows but does not prevent decarboxylation; consequently hydroxy-amino-acids are attacked. Only the natural isomers of the amino-acids are decarboxylated.

Table 1. *Specific amino-acid decarboxylase preparations for estimation of amino-acids*

Enzyme	Organism (N.C.T.C. no.)	Conditions of culture: Medium	Conditions of culture: Temperature	Conditions of culture: Time (hr.)	Specific preparation	Activity maintained	Manometric estimation: Buffer	Manometric estimation: CO_2 evolution as % theory
l-(+)-Lysine decarboxylase	*Bact. cadaveris* (6578)	3% casein digest 2% glucose (1 l. medium = 50 estimations)	25°	24	Acetone (5 vol.)-ether-dried powder, kept 3 days at 0° before use Use *c.* 10 mg. per test	4–6 weeks in desiccator	M/5 phosphate, pH 6·0 +acid-tip	92 98
l-(+)-Arginine decarboxylase	*Esch. coli* (7020)	3% casein digest 2% glucose (1 l. medium = 40 estimations)	25°	24	Acetone (5 vol.)-ether-dried powder Use *c.* 10 mg. per test	4–6 weeks in desiccator	M/5 phosphate-citrate, pH 5·2	95
l-(−)-Histidine decarboxylase	*Cl. welchii*, B.W. 21 (6785)	3% casein digest 2% glucose Heart muscle (1 l. medium = 20 estimations)	37°	16	Acetone (3 vol. for 30 min.)-ether-dried powder Use *c.* 30 mg. per test	2–3 months in desiccator	M/5 acetate, pH 4·5	96
l-(+)-Ornithine decarboxylase	*Cl. septicum*, PIII (547)	3% casein digest 2% glucose Heart muscle (1 l. medium = 20 estimations)	37°	16	Washed suspension of organism 20–30 mg. dry weight cells/ml. Use 0·5 ml. per test	24 hr.	M/5 phosphate-citrate, pH 5·5 +acid-tip	92 98
l-(−)-Tyrosine decarboxylase	*S. faecalis* (6783)	3% casein digest 1% glucose 0·1% marmite (1 l. medium = 50 estimations)	37°	16	Acetone (5 vol.)-ether-dried powder Use *c.* 10 mg. per test	2–6 weeks in desiccator	M/5 phosphate-citrate, pH 5·5	96
l-(+)-Glutamic acid decarboxylase	*Cl. welchii*, S.R. 12 (6784)	3% casein digest 2% glucose Heart muscle Hydrogen (1 l. medium = 30 estimations)	37°	12	Washed suspension of organism 20–30 mg. dry weight cells/ml. Use 0·5 ml. per test	48 hr. in H_2	M/5 acetate, pH 4·5	98

(2) *The action must not be altered by the presence of other amino-acids or nutrient materials.* A mixture of amino-acids of known composition has been made up, subjected to the usual hydrolysis procedure and the six amino-acids estimated by the decarboxylases. Secondly, assays of the six amino-acids have been carried out on hydrolysates of standard proteins and comparative assays carried out on the same hydrolysates by established methods. The satisfactory results of these tests provide further proof of the specificity of the methods.

(3) *The quantity estimated must bear a constant relation to the amount of substrate present.* The last column of Table 1 gives the experimental CO_2 evolution expressed as % theoretical decarboxylation of substrate. The decarboxylation is quantitative within the limits imposed by the CO_2-retention of the enzyme-buffer-hydrolysate system and this retention can be estimated by the acid-tip manometric method (Woods & Clifton, 1937). The values quoted were obtained as the mean of 20–30 estimations in each case and the accuracy of their application to hydrolysates etc. is shown by the results obtained with hydrolysates of known constitution. The method cannot allow for losses introduced during hydrolysis due to racemization or the presence of carbohydrate (Tristram, 1939).

(4) *The blank estimation should be negligible in comparison with the assay estimation.* The non-viable decarboxylase preparations have no blank activity. The washed suspension preparations rarely have a small but negligible gas output which is easily estimated in a blank control.

Disadvantages of the method are: (*a*) the *laevo*-isomers only are estimated, consequently racemization during hydrolysis tends to reduce results below those obtained by non-biological methods. Of the amino-acids estimated by this method, racemization during normal hydrolysis procedures either in the pure state or in protein mixtures is negligible for lysine, histidine and arginine and amounts to approx. 5 % for tyrosine and glutamic acid; (*b*) hydroxy-amino-acids are estimated; this is mainly of importance in lysine assays of gelatine, etc., where hydroxy-lysine is present to a significant extent. As the decarboxylation of hydroxy-lysine is slow, end-points are sometimes difficult to determine in such cases.

Advantages of the method include: (*a*) rapidity of assay; each estimation is complete within 20–30 mn. of mixing of enzyme and assay-material so that, with the usual Warburg outfit, it is possible to carry out 24 separate estimations per day with ease; (*b*) small amounts of assay-material are needed: accurate assays being obtained from 0·5 to 2·0 mg. amino-acid; (*c*) the method compares favourably with other procedures for accuracy and duplicate assays agree within 2 %; (*d*) the method can be applied to unpurified material such as crude hydrolysates and can therefore be used as a check on other procedures involving purification of the material prior to assay.

Other decarboxylase systems are known (Virtanen & Laine, 1937) but specific preparations have not as yet been worked out for assay purposes.

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Estimation of Amino-Acids by the Microbiological Technique. By T. F. MACRAE

Development of the microbiological assay technique for the estimation of amino-acids began only about three to four years ago, although Fildes and his colleagues had shown in 1939 that glutamine was an essential nutrient for *Streptococcus haemolyticus* and other micro-organisms (McIlwain, Fildes, Gladstone & Knight, 1939; Fildes & Gladstone, 1939). Since about 1943 there have been published, mainly in America, a very large number of papers on this subject, and the interest appears still to be increasing. At present methods are given for the estimation of all the nutritionally essential amino-acids and some of the non-essential; in all, sixteen of the amino-acids may be estimated by the microbiological technique.

The general procedures employed resemble those used in the vitamin assays. Ideally, the basal medium should be devoid of the amino-acid under investigation but complete in other ways. In the absence of the amino-acid no, or very little, growth should occur, whereas addition of the amino-acid should give a maximum growth rate. Generally, the media are composed of pure amino-acids, vitamins and other ingredients, but in the case of tryptophan assays, the amino-acids may be supplied as acid hydrolysates of proteins which, of course, contain very little tryptophan.

The micro-organisms employed include *Lactobacillus arabinosus* 17/5, *L. casei* ϵ, *L. delbrückii*, *L. fermentum* 36, *Leuconostoc mesenteroides* P60, *Streptococcus faecalis* R, and *Neurospora crassa*. Generally speaking, production of acids is the criterion employed, but sometimes growth is estimated by estimation of turbidity. In estimations using *N. crassa*, the mycelium is weighed.

The advantages of the microbiological method of assay are obvious. Compared with chemical isolation, which is the most common alternative method, the technique is simple and less time-consuming. In the case of certain amino-acids, no other method of estimation is possible. There is no doubt that the microbiological assay of amino-acids has increased our knowledge of protein composition. The technique will most certainly be used more and more in the future.

In the estimation of relatively pure amino-acids, the microbiological technique gives values which are consistent and accurate. They may be used with every confidence to determine the degree of purity of samples of amino-acids, and we have been using this technique in our laboratories with complete satisfaction.

In the estimation of amino-acids in proteins and in foodstuffs, very great difficulties, however, arise. There are many factors which influence the growth rates of the various micro-organisms. The concentration of the various vitamins, amino-acids, salts, and other nutrients, can all affect the rates of growth. Some substances inhibit growth, others increase it. Thus, when a hydrolysate, even of a pure protein, is added, the amino-acids, other than the one under test, may well have an effect. When dealing with foodstuffs containing a wide variety of substances, the possibilities of interference are obvious. It seems that a very great deal more work must be done before much reliance can be placed on the values obtained for the amino-acid contents of complex materials.

There appears to be little information on the effects of peptides on the growth of micro-organisms. It is not known to what degree hydrolysis of proteins must be carried. In favour of the microbiological technique, however, it must be stated that the values obtained for the amino-acid contents of various proteins do agree reasonably well with values obtained by chemical means.

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The Microbiological Estimation of B Vitamins. By T. F. MACRAE

The importance of B vitamins in the nutrition of micro-organisms has long been recognized. Indeed, several of the B vitamins were first recognized as essential nutrients for micro-organisms, and later proved to be essential for animals. This is the case with nicotinic acid, pantothenic acid, folic acid and inositol, in particular. Honours in respect to biotin should probably be shared by the biologist and the microbiologist.

It is now more than ten years since methods for the estimation of aneurin using *Phycomyces blakesleeanus* and yeast were developed. However, the classical work of Snell & Strong (1934), which stimulated many other investigators, appeared about seven years ago. Since then the rate of development in this field has been rapid, and the position is now that we can estimate all B vitamins, including aneurin, riboflavin, nicotinic acid or its amide, pyridoxine, pantothenic acid, biotin, folic acid, *p*-aminobenzoic acid and inositol. As with amino-acids, a whole variety of micro-organisms, including mutants of moulds, may be employed. The *Lactobacilli arabinosus* and *casei* ϵ, however, are the most favoured. To illustrate the micro-organisms which may be used, perhaps indication of what we use in our laboratories would be of value. Aneurin is estimated using *Lactobacillus fermentum* 36 and by the yeast fermentation method. Nicotinic acid, pantothenic acid and biotin are estimated, using *Lactobacillus arabinosus*. *Lactobacillus casei* ϵ is used for riboflavin and folic acid. For pyridoxine we employ *Neurospora sitophila* mutant 299. For inositol we use two yeasts. *Streptococcus faecalis* is also used for the estimation of folic acid.

We have not estimated *p*-aminobenzoic acid in our laboratories, but *Leuconostoc mesenteroides* P 60 may be employed.

The basal media for vitamin assays are on the whole simpler than those employed for amino-acids, because protein hydrolysates, provided they are prepared in such a way as to avoid contamination with B vitamins, are satisfactory sources of most amino-acids. It is, however, essential to supplement acid hydrolysates with tryptophan and cystine.

Many difficulties are encountered in the microbiological assay of the B vitamins, which do not appear to have been given as much consideration as is their due. Most vitamins are present in biological materials in only small amounts. Some factors are easily adsorbed and are rather unstable compounds. Consequently it is no simple matter to extract the vitamins quantitatively.

Growth-stimulatory effects are encountered. The unsaturated fatty acids and starch have well-defined stimulatory actions for *Lactobacillus casei* ϵ, and care must be taken to ensure removal of these materials. It is most probable that unknown essential nutrients for at least some of the micro-organisms employed also exist.

Growth-inhibitory substances are also apparent. Some salts have definite inhibitory effects under certain conditions.

Our experience with the B vitamins, however, does indicate that the microbiological technique has very considerable merit. It is particularly applicable in physiological work where materials of a similar nature are studied. It is felt that the results on any one type of biological material such as blood, urine and faeces, are at least comparable, although the values obtained may not be absolute.

A further difficulty with the vitamins is our lack of knowledge of the effects of analogues. It has been very clearly shown, for example, in the case of pyridoxine, pyridoxal and pyridoxamine, that these substances all have biological activity, but that the activity varies with the micro-organism employed. For instance, with *Saccharomyces carlsbergensis*, all three compounds show comparable activity. With *Saccharomyces cerevisiae*, pyridoxamine has only 40% of the activity of pyridoxine, and pyridoxal 46%. With *Streptococcus faecalis*, pyridoxine is inactive, whereas with pyridoxal and pyridoxamine, there is activity. In the case of *Lactobacillus casei* ϵ, pyridoxal is the only material active.

Our knowledge concerning the analogues of the other vitamins is less complete. There is too little quantitative information on the relative activities of the nicotinamide-containing coenzymes, nicotinamide and nicotinic acid. These substances are converted to nicotinic acid for determination. This makes this method of assay of less value in the assessment of the nutritive value of foods, for in the foods there is little free nicotinic acid, and nicotinamide and the codehydrogenases are by no means nutritionally equivalent to the free acid. Similarly, the effects of the riboflavin-containing coenzymes with riboflavin or riboflavin phosphoric acid are not fully investigated.

The agreement between biological, microbiological and chemical methods of estimation have been most encouraging, and although a great deal more work must be carried out, there seems little doubt that this technique will find a most important place in the assay procedures available for the estimation of the B vitamins.

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Genetic Techniques in the Development of Microbiological Assays. By G. Pontecorvo

As pointed out by Knight in the present meeting, though certain basal bio-synthetic processes seem to be common to the widest variety of organisms, species and strains differ in their ability to carry out these syntheses all the way from inorganic substances. Some therefore require as essential nutrilites organic substances which for others, that can synthesize them, are intermediate metabolites. It is not generally appreciated that similar differences in synthetic abilities do also occur between individual cells of one strain of micro-organisms. Mutant cells showing one or other specific nutritional requirement, not shown by the population among which they occur, arise repeatedly, though in general with extremely low frequencies (10^{-8} or less). If isolated such cells may give origin to a mutant strain showing permanently the new nutritional requirement.

In those micro-organisms like yeasts and certain moulds in which a regular sexual cycle exists a genetical analysis of these nutritional requirements in mutant strains has been possible by means of usual genetic techniques. The results (Beadle, 1945) are that almost always in a mutant a specific step in a chain of synthetic reactions fails to be preformed, presumably because of absence or inactivity of the specific enzyme catalyzing the step; and that the presence or absence of the active enzyme behaves in heredity as if determined by a single pair of allelomorphic genes. If the chain of reactions blocked is one involving the synthesis of an essential compound, growth of the strain may be conditional to the availability of this compound in the nutrient medium.

Fortunately, Muller's classical discovery that X-rays can enormously increase the rate of mutation, the subsequent finding that this is a property of all ionizing radiations (ultraviolet, neutrons, etc.), and the recent discovery (Auerbach & Robson, 1946) that sulphur and nitrogen mustards, as well as other chemicals, are also powerful mutagens, offer the means of producing nutritional mutants with practicable frequencies (up to 10^{-3} for each kind of mutant). It has therefore been possible, by means of irradiation, to obtain in the moulds *Neurospora* (Beadle & Tatum, 1946) and *Ophiostoma multiannulatum* (Fries, 1945) a large array of mutants, each requiring a particular water soluble nutrilite (not required by the parent strain) which may be an amino-acid, a vitamin, a purine and others, or one or more intermediates in the synthesis of one such compounds. Furthermore, identical results have been obtained with micro-organisms in which sexual reproduction of a standard type is not known to occur: e.g. in Bacteria (Tatum, 1945; Demerec & Latarjet, 1946; Devi, unpublished), in *Penicillium notatum* (Pontecorvo, 1946); in

Aspergillus terreus (Raper, Coghill & Hollaender, 1945). Implications of a fundamental nature are suggested by this parallelism of results between organisms which have hereditary particles (genes) linearly organized into chromosomes and organisms, like the smaller bacteria, where such an organization is unknown to exist and rather improbable. However, for the present purpose it is sufficient to recognize that hereditary blocks of specific metabolic steps can be induced by means of irradiation (and other treatments) in all micro-organisms; that permanent strains with such blocks can be isolated; and that, if the block involves the synthesis of a vitamin, amino-acid, etc., growth of the strain may become quantitatively dependent on the amount of that substance present in the medium.

As far back as 1941, Beadle & Tatum (1941) pointed out that mutant strains with the latter characteristics could be used in biological assays, and, in fact, assays making use of some of their *Neurospora* mutants have been developed, and one (that for pyridoxine) is at present in general use. However, because of the length of its growth period and the necessity of weighing the dried mycelium *Neurospora* is not as favoured by assayists as, e.g., acid forming bacteria. Accordingly, mutant bacteria with specific nutritional requirements are at present being produced both in this country and in the U.S.A., and some of the mutants may form the basis for the development of assays.

The possibilities and advantages of the deliberate production of mutants, rather than the use of 'natural' strains, with the necessary nutritional requirements, are the following: (1) the field of microbiological assays may be extended to new substances, for which no known 'natural' strain has a specific requirement; (2) a single organism, with its array of mutants each requiring a particular substance, can be used for many assays, thus greatly unifying the procedure; and (3) since the specificity of the requirement (i.e. requirement for one and only one substance) is a function of the position in the chain of reactions where the block occurs, mutants may be obtained with as specific a growth response as biologically possible.

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Methods for the Assay of Antibiotics. By N. G. Heatley

Chemical methods of estimation are available for only very few antibiotics, but in any case biological methods of assay (1) usually are far more sensitive, (2) are more specific, and (3) will work satisfactorily in solutions containing a variety of other substances (e.g. body fluids can be assayed directly, without any preliminary purification).

They depend on measuring whether, or to what extent, the growth or other observable function of a test organism is interfered with by a known concentration of the substance to be tested. For accurate work it is essential that the unknowns should be compared with, and expressed in terms of, a standard preparation of the *same substance*. If this is done, the result will be independent of the kind of test organism employed or the way in which the test is carried out. (Commercial penicillin may contain one or more of at least four slightly different penicillins and it is, therefore, necessary somewhat to restrict the conditions of the test.)

Many methods—chiefly for penicillin, but applicable to most other antibiotics—have been described recently but as yet no ideal method has been evolved—nor does this seem likely. Depending on the purpose for which the assay is needed, the chief requirement may be accuracy, sensitivity, simplicity, speed of setting up, speed in obtaining an answer, or specificity. The methods available excel in one or more particular, and it is important to choose the right method for the purpose in hand.

They fall into three main groups. Space permits no more than a brief catalogue of some of the chief types of method which have been used, viz.

(1) *Dilution methods.* Decreasing concentrations of the material to be tested and of the standard are set up in a suitable liquid or solid nutrient medium. The dilutions are inoculated with the test organism(s), and incubated. In the classical procedure the end-point is taken as the lowest concentration which will just prevent the growth of the test organism. In liquid media, several other end-points have been used, e.g.

(*a*) Suppression of turbid as opposed to sedimentary growth.
(*b*) Suppression of growth (turbidity) to, for example, 50 % of that in the control tube.
(*c*) Suppression of haemolysis (when the test organism is haemolytic).
(*d*) Suppression of change of pH, as shown by an internal indicator.
(*e*) Suppression of reduction of an oxidation-reduction indicator.
(*f*) Complete sterilization of the medium.

When carried out on solid medium, e.g. on a series of plates, the method is less accurate, but has the advantages that absolute sterility is not essential, and that several test organisms can be streaked on the same set of plates.

(2) *Diffusion methods.* In these a solid culture medium is seeded with a suitable test organism either in bulk or on the surface, and the solution to be assayed is brought into contact with a limited area of it. The medium is incubated, and the *distance* to which an inhibitory concentration of the substance diffuses—as shown by no growth or diminished growth of the test organism—gives a measure of the concentration of the inhibitor. By setting up at the same time different known concentrations of the substance a curve relating distance to concentration can be constructed, from which the concentration of the unknowns can be read off.

The methods fall into two groups, depending on whether diffusion occurs vertically, e.g. from a volume of test solution placed on top of a column of inoculated agar in a test-tube, or horizontally, as in a Petri dish. In the first case the organism must be a facultative anaerobe, and the depth of agar in which growth has been suppressed is measured. In the second method, which is much more generally used, a drop of the material to be tested is simply placed on the agar surface, or is deposited in a depression or cup in the agar, or in an open-ended cylinder, or on a disk of filter paper or filter fabric resting on the surface of the agar. The cup-shaped depression may be made in the agar either by embedding a marble, rubber stopper, etc., while the agar is molten, or by removing a disk from the solidified agar by means of a sterile cork borer. In all these methods the inhibitor diffuses outwards and after incubation a circular zone is seen where the test bacteria have not grown. The diameter of this zone is measured and used in the calculations. Many variations and improvements have been described, both in technique (e.g. devices for automatically placing cylinders on the plates, the use of large rectangular dishes in place of Petri dishes, special devices for measuring the zones of inhibition), and in methods of calculating the results (e.g. a four-point method is in general use in which the potency of the unknown relative to the standard is read off from a chart, while from a nomogram the error of the determinations, based on internal evidence, is derived).

(3) *Turbidimetric methods.* These depend on the fact that over a certain range of concentration, the amount of growth of the test organism, as measured by turbidity, decreases as the concentration of the antibiotic increases. A growth-concentration curve

is constructed with known amounts of a standard preparation, and from it, the concentration of the unknowns (set up at the same time) are read off. These methods may give good results under defined conditions, e.g. on batches of material during manufacture, or on blood serum from fasting patients, but they are liable to be upset by non-specific inhibitors or stimulators of the test organism.

(4) *Miscellaneous methods.* The plate-droplet method of Bruce White for the standardization of penicillin has given good results over a considerable period of time, though it is less useful where large numbers of assays have to be run, or where the approximate potency of the samples to be assayed is not known.

Manometric measurement of their effect on the gaseous metabolism of bacteria affords a very laborious method of assay of antibiotics, but is valuable where slow-growing test organisms such as the tubercle bacillus must be used.

Some antibiotics have a powerful effect (which reaches its optimum after about 30 min.) in abolishing the luminescence of certain luminous bacteria, an effect forming the basis of a rapid method of assay. As antiluminescent effect is unrelated to bacterial inhibitory effect, the method is completely unspecific. It has, however, been useful in the study of aspergillic acid, and may be applicable to other substances also.

The reduction of nitrate to nitrite by living staphylococcus is diminished to an increasing extent by increasing concentrations of some antibiotics. The nitrite production after about 1 hr. is estimated colorimetrically after diazotization, and the concentration of antibiotic in the unknowns is read off from a curve relating nitrite production to concentration, obtained under the same conditions with solutions of known potency.

When the substance to be assayed has a lytic or haemolytic action, it can be rapidly assayed by the classical procedure of finding the least concentration which will just cause lysis under certain conditions.

Other effects which have been used for the assay of antibiotics are: rate of killing of sensitive bacteria, degree of protection conferred on experimentally infected animals, and effect on fungus spore germination count.

It is impossible to discuss or even mention the advantages and disadvantages of each type—which give a rapid answer, which must be avoided if the solution is not sterile, the time taken in setting up, the most convenient test organisms, the accuracy to be expected, etc. It is, however, perhaps worth mentioning that in assays of the graded response type (e.g. turbidimetric, diffusion) the probable error can readily be calculated, and it is a straightforward procedure to increase the accuracy to any desired degree by setting up sufficient replicates. The dilution methods are of the all-or-none type, and the answer is given not as a definite value, but as a range. The range may be narrowed by setting up dilutions in smaller steps, but unless certain media are used, the end-point becomes correspondingly obscure. Diffusion methods are by no means universally applicable; they are not as sensitive as some methods, and cannot therefore be used with almost insoluble substances, or for estimating low concentrations, e.g. as in blood; with some substances, too, the concentration-zone diameter curve may be too flat for accurate assay.

The Assay of Antitoxins. By C. L. Oakley

In bacterial infections of animals, the whole or part of the disease process can often be confidently ascribed to the action on the cells or tissues of the host of soluble substances of high molecular weight secreted by the infecting organisms. These substances, the bacterial 'toxins', can usually be obtained free from bacteria but associated with much inactive material by passing fluid bacterial cultures through bacteria-proof filters. The filtrates so obtained are often called 'toxins', but as they are often complex, it is better to reserve the word 'toxin' for the separate toxic components of the filtrate. They are a heterogeneous collection; a few are certainly enzymes—e.g. the α-toxin of *Cl. welchii*,

the β- and γ-toxins of *Cl. oedematiens* are lecithinases splitting lecithin into phosphocholine and stearyloleylglyceride (Macfarlane & Knight, 1941; Oakley, Warrack & Clarke 1947); the K-toxin of *Cl. welchii* is a collagenase (Oakley, Warrack & van Heyningen, 1946). The nature and mode of action of almost all the rest is unknown, and their presence is determined by the indicating effects they produce. Some, for instance, are lethal on intravenous injection, some produce a necrotic lesion, some a bright red flush, on intracutaneous injection; some are haemolytic. All are antigenic, i.e. on injection in proper amounts into living mammals they excite the production of antitoxins capable of neutralizing their toxicity. It is obviously useful to have methods of evaluating antitoxins, and the use of bacterial toxins for this purpose may be regarded as a form of microbiological assay. The high specificity of the toxin-antitoxin reactions on which these tests depend need not be emphasized here. It might be supposed that an easy way of finding the value of an antitoxin would be to determine the amount of antitoxin capable of neutralizing say 100 effective doses (e.g. lethal or haemolytic doses) of the toxin concerned. In practice the varying amount of toxoid present in toxic filtrates renders this method unreliable, for toxoid combines with antitoxin but has no direct indicating activity; all antitoxins are therefore evaluated in terms of an arbitrary standard by determining the amount of unknown serum which will under proper conditions reduce the activity of the 'test dose' of filtrate to some specified level. The test dose is that amount of toxic filtrate which, mixed with x units of standard antitoxin, produces under suitable conditions a standard effect on the indicator. The level of test (x units) should be chosen so that the test dose contains a fair number (say 30) of indicating doses of toxin. Any convenient indicating effect may be used. Where the toxin is an enzyme, it may be the liberation of a certain amount of a well-defined chemical substance from its substrate—e.g. the liberation of water-soluble phosphorus from lecithin by the α-toxin of *Cl. welchii*. In appropriate cases 20% haemolysis of a 6% suspension of susceptible red cells, or the production of a necrotic area 2×2 mm. in the guinea-pig's skin, or death of half the animals injected, or the production of tetanus in $3\frac{1}{2}$ days, may be used.

The test dose of toxic filtrate so determined is then mixed with varying amounts of the unknown serum; that mixture which under the standard conditions produces the standard indicating effect contains x units of antitoxin. By this means the amount of antitoxin in any serum in relation to a standard is readily determined. The usual statistical precautions must of course be taken.

One method of titration—the flocculation reaction—differs from all the others in that no external indicator is required, thus avoiding the complications introduced into other tests by competition between indicator and antitoxin for the toxin. It suffers, however, from the difficulty of determining without more investigation which is the specific zone of flocculation.

Several things are worth noting. If, as is usually the case, a toxin has numerous indicating effects, serum values against the toxin using these effects should be the same. Conversely if the serum values against a filtrate using several different indicating effects are the same, it is probable that one toxin is responsible for them all. If serum values against a filtrate using different indicators are different, it is probable that the filtrate is complex and contains more than one toxic substance. If only one toxin is present in numerous batches of toxic filtrate obtained from the same organism, serum values against all the batches using the same indicator should be the same; if they are not, a possible explanation is that the filtrates contain, in varying proportion, more than one substance capable of affecting the indicator.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **254th Meeting** *of the Biochemical Society was held at the National Institute for Medical Research, Hampstead, London, N.W. 3, on Friday, 6 December* 1946, *at* 1.30 *p.m., when the following papers were read:*

COMMUNICATIONS

Clinical Assay of Vitamin P. II. The Dose/Response Curve for Hesperidin. By H. SCARBOROUGH and F. WOKES. (*Clinical Laboratories of the Royal Infirmary, Edinburgh, and the Ovaltine Research Laboratories, King's Langley, Herts.*)

Determination of capillary resistance (C.R.) in boys under controlled conditions have shown that the response to dosing with preparations of vitamin P is affected by the initial C.R. (Scarborough & Wokes, 1946). Factors for adjusting to a standard initial C.R. of 300 mm. Hg are now shown not to differ significantly at different dosage levels and a set of composite factors is described which have given satisfactory results in a long series of experiments. To obtain a dose/response curve for hesperidin nine equal groups, each containing five boys, were studied. Three of these groups (fifteen boys) received only the control diet through the experiment. The six test groups were fed purified hesperidin (supplied by Glaxo Laboratories, Ltd.) for 5 days to give total dosages of 375, 500, 625, 1125 and 1250 mg. The C.R. of all boys was determined in each of two standard areas at the beginning and end of the 5-day period. After factorization the results in the two areas were pooled and by making due allowance for the effect of the standard diet on the controls a dose/response relationship was determined by plotting log. response against log. dose. The relationship proved to be linear within the range 500–1000 mg. on the log. dose scale.

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Proteolysis of Cartilage and Bone (Loss of Calcium). By P. PINCUS. (*Pathology Dept., Medical School, Westminster Hospital, S.W.* 1)

Loss of tooth structure occurs in dental caries. Part of this loss must be due to proteolysis since protein forms the organic matrix of dentine; protein also forms the organic matrix of cartilage and bone. Bacteria capable of proteolysis have long been described in the mouth: *B. proteus*, *F. necrophorus* (Topley & Wilson, 1936), *B. perfringens* (Glynn, 1923).

In the present series of experiments, routine bacteriological methods have been applied to the isolation of proteolytic bacteria from carious human teeth. Cartilage, which had been sterilized by being kept for an hour on each of eight successive days at 57° C., was exposed to pure cultures of one of these organisms, a Gram-negative bacillus. This organism resembles *B. proteus* in several cultural characteristics. In later experiments sterile cartilage was exposed to Seitz filtrates of broth cultures of the same organism. It was found that lysis of cartilage took place, the white of the cartilage in a few days turning to yellow and then to amber, giving as an end result, in 2–3 weeks, a brownish liquid. Experiments were carried out at 37° C.

Sterile bone (cortex from the femur of a cow aged about 5 years) was next exposed to cultures of the same Gram-negative bacillus. After 4–6 weeks, the cubes of bone showed evidence of attack by the bacteria and were reduced to a fragmentary condition. The experiments were then extended to the exposing of sterile bone to Seitz filtrates of broth cultures, the same Gram-negative bacillus being used throughout. While attack has occurred less frequently with the Seitz filtrates than with the cultures, in some cases the complete breakdown of the bone has been seen with the filtrates.

Frequent estimations of pH values have been made, using indicators. Values ranged from pH 7 to 8, sometimes nearer 8·5.

While heating rib-bones from day-old calves in broth at pH 7·5, it was noticed that the bones lost

calcium. Estimations of calcium were carried out on the bone after heating it at 100° C., to constant weight and then calcining, and on the supernatant liquid, by oxalate precipitation. It was later found that bone may lose calcium to physiological saline; in some cases as much as 20 % of the calcium present in young rib-bone may pass into the surrounding liquid, on heating once to 100° C., for 1 hr.

These experiments provide some evidence that bone may lose calcium to fluids with pH values close to 7. Proteolysis, either by pure cultures, or by sterile filtrates of pure cultures, may be followed by release of calcium. A detailed account of methods used, with photographs of 'lysed' cartilage and bone, and the results of the calcium estimations, is to be published.

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Folic Acid and the Growth of Insects. By G. FRAENKEL and M. BLEWETT. (*Imperial College of Science and Technology, Biological Field Station, Slough, Bucks.*)

The rate of growth of three different insect species (*Ephestia kuehniella*, *Tenebrio molitor* and *Tribolium confusum*) on an artificial diet which contains all the known factors of the vitamin B-complex in pure substance is markedly accelerated by the addition of synthetic folic acid (Lederle) (Fraenkel & Blewett, 1946). With *Ephestia*, vitamin B_c (Parke, Davis & Co.) has approximately the same effect, and xanthopterin has a comparable effect in concentrations about 1000 times greater. With *Tenebrio*, if in addition to the known vitamins the diet also contains a folic acid free extract from yeast or liver, folic acid becomes the only limiting factor of growth. Xanthopterin appears to have no effect on *Tenebrio*. With *Tribolium*, a folic acid deficiency has been demonstrated on certain purified preparations of casein, but not on others. It is concluded that different samples of 'vitamin free' caseins differ widely in their folic acid content.

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The Effect of Linoleic Acid and Arachidonic Acid on the Development of the Flour Moth, *Ephestia kuehniella*. By G. FRAENKEL and M. BLEWETT. (*Imperial College of Science and Technology, Biological Field Station, Slough, Bucks.*)

We have previously shown that the moths of the genus *Ephestia* require linoleic acid and α-tocopherol in the diet, the former for normal development of the wings and successful emergence, and the latter as an antioxidant for linoleic acid and for rapid growth (Fraenkel & Blewett, 1946 *a*, *b*). Since then arachidonic acid has been tested and found to have no effect whatsoever on the wings and emergence, but a very striking positive effect on growth. In both respects its effect resembles very closely that of docosahexenoic acid from cod-liver oil.

In order to find out whether linoleic acid is synthesized in insects, two insects, one which requires linoleic acid (*Ephestia*) and one which does not (*Tenebrio*), were grown in the presence and absence of linoleic acid. The linoleic acid content of the oils from these insects was determined by testing their effect on the wing development of *Ephestia*. The oils from both insects, grown on a diet with linoleic acid, contained roughly 9–12 % linoleic acid. Of the insects grown in the absence of linoleic acid, the oil of *Tenebrio* seemed to contain roughly 6 % linoleic acid, but that of *Ephestia* less than 1½ %. It is concluded that linoleic acid is synthesized by *Tenebrio* but not by *Ephestia*.

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Flocculation Tests with Electrophoretically Separated Serum Proteins. By N. F. MACLAGAN and D. BUNN. (*Shrubsall Biochemical Laboratory, Westminster Hospital Medical School, Horseferry Road, London, S.W.* 1)

A study of the electrophoretically pure fractions from four collections of human serum has given the following results:

1. The thymol turbidity test (Maclagan, 1944*a*) gave a faint turbidity with γ globulin fractions, producing, however, only about 1 unit of turbidity per mg. of protein.

2. Addition of cephalin or lecithin to the γ fractions produced a much increased thymol turbidity, amounting to 10–15 units per mg. This turbidity was abolished by normal albumin in the proportion of 4 parts of albumin to 1 of γ globulin, but was not reduced by hepatitis albumin.

3. The colloidal gold reagent (Maclagan, 1944*b*, 1946) was precipitated by γ fractions only, albumin being inhibitory in proportion of 10 : 1. α and β fractions were much more strongly inhibitory, in the proportion of about 2 : 1. The sensitivity for γ globulin varied from 0·075 mg. at pH 7·8 to 2 μg. at pH 6·6.

4. The cephalin cholesterol reagent (Hanger, 1939) was flocculated by normal γ fractions (0·5 mg.) but much more strongly by hepatitis γ globulin (0·05 mg.) and hepatitis ($\alpha+\beta$) globulin (0·06 mg.). Albumin was inhibitory to both fractions in the proportion of 100 : 1.

5. The chief differences between normal and hepatitis fractions were:

(*a*) Hepatitis γ globulin was more effective than normal γ against gold (at pH 7·8) and cephalin cholesterol reagents, the respective sensitivities being 0·025 and 0·075 mg. for the gold and 0·05 and 0·5 mg. for the cephalin cholesterol.

(*b*) Hepatitis ($\alpha+\beta$) fractions flocculated the cephalin cholesterol reagent, normal α and β did not.

(*c*) Normal albumin inhibited the thymol-cephalin reaction, hepatitis albumin did not.

(*d*) Hepatitis albumin and ($\alpha+\beta$) fractions increased the turbidity of the γ globulin with thymol in the absence of added cephalin, normal albumin α and β fractions did not.

It appears therefore that all four tests depend upon slightly different mechanisms, and that qualitative as well as quantitative changes in the electrophoretic fractions are important. The thymol-cephalin test approaches closest to being simply a test for relative gamma globulin excess. This is presumably the same as the original thymol turbidity test as applied to serum, the native serum lipids participating in the reaction as stated previously (Maclagan, 1944*a*). In support of this addition of cephalin to a number of normal and hepatitis sera did not appreciably increase the thymol turbidity. A γ-globulin-lipid complex responsible for the thymol test is possibly disrupted by electrophoresis but can be re-constituted by addition of lipids to the γ fraction.

These results differ in certain important respects from those of other workers (Moore *et al.* 1945; Recant *et al.* 1945).

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The Fate of Nicotinamide Methochloride in the Rat. By P. ELLINGER. (*Lister Institute of Preventive Medicine, Chelsea Bridge Road, S.W.* 1)

Nicotinamide methochloride (NM) is eliminated in human urine when ingested orally to about 15% and after parenteral application to about 60% (Ellinger & Coulson, 1944; Perlzweig & Huff, 1945). The remainder is unaccounted for. Perlzweig, Huff & Rosen (1946) believe in a 'destruction', Najjar & Deal (1946) in a demethylation of NM, both processes taking place in the liver.

To test this view:

(*a*) Rat liver slices of 0·2–0·4 mm. thickness and 300–500 mg. wet weight were agitated at 37° in 10 ml. Ringer solution (Krebs & Henseleit, 1932) containing 1, 3, or 10 μg/ml. NM. The centrifuged liquid was tested for NM (Coulson, Ellinger & Holden, 1944), after 2, 30, 60, 120 and 240 min. shaking. In blanks without liver there was no significant loss after 4 hr. In the samples containing liver there was a loss after 2 min. of 12%, 7%, and 3% in the samples containing 1, 3, and 10 μg./ml. NM, respectively. No further significant loss occurred during the next 4 hr.

(*b*) Rat livers were perfused either from the liver artery under 90 cm. water pressure or from the portal vein under 40 cm. water pressure at 37° with

Krebs-Ringer containing 1, 3, or 10 μg./ml. NM. In the perfusion liquid a loss of 4–10% of NM was found after 10 min., but no further loss was observed. Both results indicate some adsorption, but no destruction of NM in the liver.

Search for the unaccounted NM was made in faeces. The results were inconclusive. In normal rats on normal diet the daily faecal output varied between 15 and 1100 μg. and after dosage of either nicotinamide or NM the output was sometimes increased, sometimes not; never more than 10% of the ingested NM was recovered from the faeces.*

To examine the effect of faecal bacteria on NM a few drops of a faecal emulsion was incubated at 37° for 4 days in ammonium lactate medium (Fildes, 1938) containing 5 μg./ml. NM. In three experiments 78·7%, 89·3%, and 92·4% NM were destroyed. A simultaneous König reaction test after hydrolysis with strong alkali (Najjar, Scott & Holt, 1943) gave no colour in the former case and showed similar results as with NM test in the latter. This proved that the bacteria destroyed the pyridine nucleus of NM. There is, however, some indication that certain intestinal bacteria form NM from nicotinamide (Ellinger & Kader, 1946.* Both destruction and formation of NM by the intestinal flora, the conditions of which are still unknown, explain the inconclusiveness of the daily faecal NM output.

Lastly, the rat bile was searched for the missing NM. It was collected from nembutalized rats through a cannula fixed in the duodenum near the bile-duct entrance. In some instances it was possible to collect bile continuously at 0·6–0·8 ml./hr. for 4–5 hr. It was tested for NM and 2·2–18·0 μg./NM/hr. were eliminated. After parenteral application of 10 mg. of either nicotinamide or NM, the elimination rose to 10·6–98·0 μg./hr. up to 4 hr. When it was possible to collect urine simultaneously the biliary output was 9–37% of the urinary elimination.*

Thus, the missing NM is probably eliminated with the bile into the intestines and there disintegrated by the intestinal flora.

* NM was identified by its reactions towards strong alkali, potassium ferricyanide and alkaline acetone (Najjar, Scott & Holt, 1943).

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The Effect of Diet on the Glutathione Content of the Liver. By G. Leaf and A. Neuberger.
(*National Institute for Medical Research, Hampstead, N.W.* 3)

It has been shown recently (Glynn *et al.* 1945) that white rats maintained on a diet deficient in cystine and methionine develop, after varying periods of time, massive necrosis of the liver. In this connexion it was thought of interest to determine whether any change in the body composition was associated with a deficiency of these amino-acids. The effect of diet on the glutathione content of the tissues was therefore studied, special attention being paid to the liver.

On a diet low in protein the glutathione content of the liver fell rapidly. The change after 1 day was statistically significant, but after 5–10 days the values reached a constant level corresponding to 20–30% of the normal value.

This fall was prevented by administration of methionine or cystine without a corresponding increase of the intake of other amino-acids. Similarly normal amounts of cystine or methionine rapidly restored the glutathione content of the liver after depletion by varying periods of protein deficiency. Other organs appeared to be much less sensitive to dietary changes than the liver in this respect and starvation appeared to have a less marked effect on the glutathione content of the liver than protein deficiency.

Increasing the intake of methionine or cystine to a higher level did not have a very marked effect on the glutathione content of the liver.

Similar results were obtained in experiments in which the dietary nitrogen was supplied in the form of pure amino-acids.

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The 'Virulence-Enhancement' Factor of Gastric Mucin. By J. C. GOULD and H. K. KING. (*Bacteriology Dept., University New Buildings, Teviot Place, Edinburgh*, 1)

The effect of gastric mucin in augmenting the virulence of pathogenic bacteria has frequently been attributed to the protective action of the viscous fluid in preventing access of the defensive mechanisms of the host. The exhibition of 'virulence enhancement' by substances such as agar, chemically unrelated but with similar physical properties, has been cited in support of this hypothesis.

The present investigation indicates that whilst agar may indeed act through affording the pathogen such physical protection, the effect of mucin undoubtedly involves some other mechanism since it is qualitatively different and quantitatively more intense. A fraction has been prepared from hog gastric mucin (Wilson, type 1701-W) in which an approximately twentyfold concentration of the 'virulence-enhancement' factor has been achieved; but solutions of this concentrate are not appreciably viscous.

Various pathogens were employed with both agar and mucin; the results with meningococcus may be taken as typical. Thus 10 mg. agar in 1 ml. reduced the mouse intraperitoneal M.L.D. by a factor of 100. The meningococci multiplied in the peritoneal cavity, overwhelming the phagocytes and producing a fatal toxaemia without, however, invading the blood stream. Under similar conditions, 5–10 mg. crude mucin or 0·25 mg. of the concentrate lowered the M.L.D. by a factor of 10,000; and a factor of 1,000,000 could be obtained with larger doses of mucin. The nature of the infection was radically different from that enhanced by agar. An early and progressively increasing bacteriaemia was observed and death resulted from septicaemia. A corresponding peritoneal proliferation was also found, but the number of organisms was fewer than in agar-enhanced infections. Mucin thus appears to contain a substance comparable with the bacterial aggressins.

The factor was concentrated by differential centrifugation. Mucin was suspended in water and centrifuged at 3000 r.p.m. A coarse sediment was deposited leaving a slightly opalescent viscous supernatant which appeared homogeneous, but was seen under the microscope to contain numerous particles of the order of size of 1 μ. These, which comprised about 10 % of the mucin, were deposited on prolonged centrifugation (1–2 hr.) at 15,000–20,000 r.p.m. and were then washed free of the viscous, inactive supernatant. The precipitate was dissolved in N/15 sodium carbonate and reprecipitated at pH 3·0. This material, representing 5 % of the original mucin, could be redissolved in alkali; excess was removed by dialysis, giving an almost neutral solution, suitable for injection and containing at least 50 % of the virulence-enhancing activity of the original mucin.

No detailed chemical investigation has yet been undertaken, since it is intended to attempt further purification. The following data suggest that the material was predominantly protein in character: N (Kjeldahl) 10·8 %; Molisch negative; biuret positive; strongly positive reactions for arginine, tryptophan, tyrosine; 80 % of the N liberated as amino-N on acid hydrolysis.

The Effect of Diet on the Succinoxidase of Rat and Mouse Livers, and on its Inhibition by Metabolic Products of Carcinogenic-Azo Compounds. By L. A. ELSON. (*Chester Beatty Research Institute, Royal Cancer Hospital, Fulham Road, S.W.* 3)

The succinoxidase activity of rat and mouse liver homogenates has been found to be greatly influenced by the diet of the animals. Variations observed in the activity of the liver succinoxidase and in its inhibition by metabolic products of carcinogenic-azo compounds (Elson & Hoch-Ligeti, 1946) have now been shown to be related to these dietary effects.

Vitamin B group. Deficiency of the B group of vitamins appears to lower the proportion of the cytochrome *c* component of the succinoxidase system, while the succinic dehydrogenase and cytochrome oxidase remain relatively unaffected. Thus, liver homogenates of mice maintained on a high (20 %) protein, high vitamin B diet show an oxygen uptake in presence of succinate of 7000–10,000 (mean 8300) μl. O_2/g. liver tissue/hr. when measured without addition of cytochrome *c*, rising to 10,000–14,000 (mean 12,000) on addition of cytochrome *c*; mice on a 20 % protein, vitamin B-free diet show activities of 2500–5000 (mean 3700) without cytochrome *c* but rising with addition of cytochrome *c* to practically the same value—10,000–14,000 (mean 11,500)—as that of animals receiving adequate B vitamins.

The result of this lowering of cytochrome *c* is reflected in the behaviour towards metabolic products of carcinogenic-azo compounds. On the high protein high vitamin B diet inhibition of the liver succinoxidase by dimethyl-*p*-phenylene diamine occurs both without and with addition of cyto-

chrome *c*, whereas with the vitamin B-deficient animals no inhibition is observed without addition of cytochrome *c*.

Protein. The main effect of lowering the protein content of the diet has been found to be a decrease in the amount of the dehydrogenase component of the succinoxidase system. The mean times of decolorization of methylene blue (Thunberg technique) by homogenates of the livers of rats on diets of varying protein content are:

25% protein (Casein-flour diet)	20% protein (Casein-flour diet)	10% protein (Casein-starch diet)	5% protein (Casein-starch diet)
5·4 min.	6·0 min.	11·0 min.	15·2 min.

On a 6% protein diet (White & Edwards, 1942) the succinoxidase activity (Warburg technique) of rat-liver homogenates was 1750–3400 (mean 2300) μl. O_2/g./hr. without cytochrome *c* and 3300–6500 (mean 4240) with cytochrome *c*, and all animals had fatty livers. Addition of methionine to the diet largely prevented fatty infiltration of the liver and showed some increase in the succinoxidase (2500–5300, mean 3870, without cytochrome *c*; 4700–8500, mean 6400 with cytochrome *c*). On the basal diet no inhibition of succinoxidase activity by dimethyl-*p*-phenylene diamine without addition of cytochrome *c* was observed, whereas on the methionine supplemented diet marked inhibition occurred both with and without cytochrome *c*.

White & Edwards (1942) showed that addition of cystine to the basal diet resulted in considerable shortening of the time of liver tumour production by *p*-dimethylaminoazobenzene, and it has now been found that if rats are maintained on the methionine supplemented diet for 12 weeks the subsequent addition of *p*-dimethylaminoazobenzene results in rapid development of tumours in all the animals.

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Di-isopropyl Fluorophosphonate as a Selective Inhibitor of Pseudo-Choline-Esterase. By B. Mendel and R. D. Hawkins. (*Banting Institute, Toronto, Canada*)

Bodansky & Mazur (1946) and Mazur & Bodansky (1946) found that the negative log of the molar concentration of di-isopropyl fluorophosphonate (DFP) at which 50% inhibition of various cholinesterase occurred, was 7·7 for human serum and 8·1 for horse serum, whereas the values for rabbit serum, erythrocytes of various species and brain ranged from 4·1 to 6·0. In view of the fact that human serum and horse serum contain predominantly pseudo-choline-esterase (Mendel, Mundell & Rudney, 1943), whereas rabbit serum contains mainly true choline-esterase (Mendel & Rudney, 1945), the variations in sensitivity reported by Mazur & Bodansky for the various 'serum cholinesterases' suggested that DFP, like the dimethyl carbamate of (2-hydroxy-5-phenylbenzyl) trimethylammonium bromide (Hawkins & Gunter, 1946), might be a selective inhibitor of pseudo-choline-esterase. *In vitro* experiments, in which acetyl-β-methylcholine and benzoylcholine were used as substrates (Mendel *et al.* 1943) to estimate the activity of true and pseudo-choline-esterase separately in plasma containing a mixture of both these enzymes, showed the DFP, in certain low concentrations (5×10^{-8}M for dog and cat plasma, 5×10^{-7}M for rabbit plasma and 10^{-6}M for rat plasma) indeed inhibits selectively the pseudo-choline-esterase, which can be dispersed with *in vivo* as far as the hydrolysis of acetylcholine is concerned (Hawkins & Gunter, 1946).

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The Role of SH Groups in the Myosin-Actin Interaction. By K. Bailey and S. V. Perry. (*Biochemistry Dept., Downing Street, Cambridge*)

The interaction of myosin and actin is revealed by a viscosity greatly in excess of either component (Straub, 1942). On addition of adenosinetriphosphate (ATP) the viscosity falls immediately to a value equal to the sum of the separate components. Since the effect is observed in salt solutions of ionic strength up to $\mu = 2$ (Szent-Györgyi, 1945), it may be inferred that the interaction is not wholly of electrostatic type, but involves a special interaction between specific chemical groupings. This inference

is supported by the finding that the SH groups of myosin, but not of actin, are essential for actomyosin formation.

The effect of specific SH reagents is shown below:

Reagent	Amount (m.mol./mg. myosin)	Inhibition of actomyosin formation
Iodoacetate	0·02	Complete
Iodoacetamide	0·006	—
o-Iodosobenzoate	0·000035	Almost complete
p-Chloromercuribenzoate	0·00004	—

Only in the case of chloromercuribenzoate is the inhibiting action reversed with cysteine.

In the case of iodosobenzoate, first used as SH-oxidant by Hellerman, Chinard & Ramsdell (1941), a method has been developed for estimating SH groups oxidized at the point of complete inhibition of myosin-actin interaction: it corresponds to 0·5% as cysteine, a figure likewise derived by assuming stoichiometric reaction between myosin-SH groups and chloromercuribenzoate at the concentration cited above. Aged myosin preparations contain no SH groups and do not react with actin.

In contrast to myosin, SH reagents have little effect upon the ability of actin to interact with myosin, and 0·8% of its cysteine may be oxidized by iodosobenzoate without influencing the reaction.

There appears a close parallelism between the concentrations of alkylating, oxidizing and mercaptan reagents preventing actomyosin formation, and those found by Singer & Barron (1944) to inhibit ATPase. A more detailed comparison is in progress.

Electron microscope studies (Astbury, Perry & Reed, unpublished) show that actomyosin forms an anastomosing network of filaments which breaks up into its component molecules on addition of ATP. It is probable that ATP competes with the actin for the myosin-SH grouping, since wherever ATP acts as substrate, it does so with enzymes of sulphydryl character, and may be deemed to have a special affinity for this grouping in proteins.

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The Action of Proteolytic Enzymes on Insulin. By J. A. V. Butler, E. C. Dodds, D. M. P. Phillips and J. M. L. Stephen. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.* 1)

A preliminary study has been made of the action of crystalline pepsin, chymotrypsin and trypsin on insulin. Contrary to numerous statements in the literature, trypsin was found to have no appreciable action. Insulin was recrystallized almost quantitatively from a solution in which it had been exposed to the action of trypsin for 18 hr. and was found to be identical in biological potency with the original.

With pepsin at pH 2, rapid hydrolysis occurs to 12% amino-N, the products being not precipitated by 5% trichloroacetic acid and able to diffuse through cellophane. Further change is very slow. At this point fourteen free NH_2 groups are present in the submolecule of mol.wt. 12,000, as compared with four in the original insulin, i.e. ten peptide bonds have been broken. The average molecular weight of peptide chains in the digest is thus about 860, corresponding to seven or eight residues. The digest on neutralization deposited some insoluble material which is being further investigated. Determinations of the diffusion coefficient on the soluble part gave $D_{20} = 1{\cdot}9 \times 10^{-6}$ cm.^{2}/sec., corresponding to an average molecular weight of 2800 ± 500. The soluble fragments produced by the digestion thus contain on the average three or four peptide chains, presumably held together by —S—S—. Not much information has been obtained about the uniformity of the digest, but the ninhydrin reaction shows that the amount of free amino-acid formed is very small, being not more than 1% of the N.P.N. liberated.

In the digestion by chymotrypsin at pH 8 there are two stages: (i) a rapid hydrolysis to about 50% N.P.N., at which point the activity has almost completely disappeared; (ii) a subsequent action in which the N.P.N. rises slowly to 100%. In the final state the free amino-N is about 15%—i.e. the digestion proceeds some 25% further than the primary stage with pepsin. The amount of free amino-acid formed is again small (2–3% of N.P.N.). A possible interpretation of all the facts is that the initial action of chymotrypsin consists of (i) splitting off of one or two terminal amino-acid groups; (ii) the splitting of four or five additional peptide bonds releasing up to 50% of the molecule which appears in the 'non-protein' fraction, the residue being precipitable by trichloroacetic acid

Conversion of Retinene$_1$ to Vitamin A *in vivo*. By S. BALL, J. GLOVER, T. W. GOODWIN and R. A. MORTON. (*Department of Biochemistry, The University, Liverpool.*)

Retinene$_1$ (vitamin A aldehyde) is now available free from vitamin A (Ball, Goodwin & Morton, 1946), and practically pure. It has been administered orally (1·5–4·5 mg.) to young rats fed on a vitamin A-deficient diet until the liver reserves of control animals were exhausted. Retinene$_1$, but not vitamin A, could be detected in the intestinal contents 5–25 hr. after dosing. Vitamin A, but no retinene$_1$, was found both in the intestinal wall and in the liver. Subcutaneous administration of retinene$_1$ results in the appearance of vitamin A but not retinene in the liver.

The data indicate that retinene given orally is unaltered until it is absorbed, and that it is quickly reduced to vitamin A in the mucous membranes of the gut and later the vitamin is transported to the liver. That the liver can perform the same reduction is shown by the fate of the injected retinene.

Quantitative experiments suggest that one molecule of vitamin A appears for each molecule of retinene absorbed, implying the existence of an efficient reductase.

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Ball, S., Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, *Proc.* lix.

Conversion of Retinene$_2$ to Vitamin A$_2$ *in vivo*. By R. A. MORTON, M. K. SALAH and A. L. STUBBS. (*Department of Biochemistry, The University, Liverpool*)

Retinene$_2$ (vitamin A$_2$ aldehyde) obtained free from spectroscopically detectable amounts of vitamins A or A$_2$ or retinene$_1$ (Morton, Salah & Stubbs, 1946) has been fed (4 mg.) to young rats depleted of vitamin A. The retinene$_2$ persists only until it enters the gut wall where it is quickly reduced to vitamin A$_2$, recognizable by its ultra-violet absorption and by the spectrum of the blue-green colour produced with the antimony trichloride reagent. Vitamin A$_2$ can also be found in the livers.

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DEMONSTRATIONS

Semi-continuous Electrophoretic Separation of Proteins. By D. BUNN. (*Dept. of Chemistry, The University, Leeds*, 2)

The yields obtainable by the Tiselius electrophoretic technique (Tiselius, 1937, 1938) can be improved upon by applying the electric potential for much greater periods of time, removing fractions periodically, and returning to the U-tube those fractions containing more than half the number of components present. This principle has been embodied in apparatus devised by Svensson (Svensson, 1942, 1946). A simpler adaptation of the standard Tiselius assembly has been used to advantage in the separation of serum proteins.

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Electron Micrographs of *Staphylococcus* Bacteriophage "K" and the Viruses of Vaccinia and Newcastle Disease. By W. J. Elford, J. Smiles, C. M. Chu and J. A. Dudgeon. (*National Institute for Medical Research, Hampstead, N.W. 3*)

Electron micrographs of specimens prepared by the usual method on collodion films (Necoloidine), with and without subsequent shadowing with gold (Williams & Wyckoff, 1945) were exhibited. The R.C.A. instrument, model B, had been used, employing 50 kV. electrons. Reproductions were enlarged threefold giving final magnification × 35,000.

(1) *Staphylococcus 'K' bacteriophage.* Lysed culture of *Staph. aureus* in Fildes synthetic medium was centrifuged to deposit the phage which was then suspended in water for E.M. examination. The gold-shadowed preparation yielded images of excellent contrast and the drumstick-like morphology of the phage was strikingly revealed, whereas on the untouched specimens the tails were but faintly visible. A point of interest in such a once centrifuged preparation is the presence of 'free' tails greatly in excess of free 'heads'.

(2) *Vaccinia virus.* Lister Institute strain. Virus, from rabbit skin, in successive stages of purification by washing in M/400 citrate-phosphate buffer pH 7, was examined. Virus bodies are attended in the early stages by material which is progressively dispersed on washing leaving the relatively clean opaque brick-like image, already described by Smadel, Anderson & Green (1942). In pictures of the gold-shadowed specimens the surface contours of the dried virus particles were clearly discernible.

(3) *Newcastle disease virus.* Doyle strain. Virus was purified from infected allantoic fluid by differential centrifugation in the air-driven ultracentrifuge. The E.M. photographs were intriguing. The images were of low contrast and indicated a peculiar pleomorphism in which sperm-like structures were observed. The tails of these sperm-like forms were often curled to form a loop attached to the head, which latter was generally of greater electron density. Gold-shadowed preparations confirmed this picture and provided the much needed contrast. How far the sperm-like images represent the true morphology of the virus, or whether they and other less well-defined elements are the result of distortions occurring during the drying of an essentially round or oval body jacketed with a substance of mucinous consistency, is as yet an open question.

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The **255th Meeting** *of the Biochemical Society was held at the British Postgraduate Medical School, Ducane Road, Hammersmith, London, W. 12 on Saturday, 25 January* 1947, *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

Clinical Assay of Vitamin P. III. Seasonal Variations in the Vitamin C and Vitamin P Content of the Experimental Diet. By A. J. Glazebrook, H. Scarborough and F. Wokes. (*From the Department of Clinical Medicine and the Clinical Laboratories of the Royal Infirmary, Edinburgh, and the Ovaltine Research Laboratories, King's Langley, Herts.*)

In order to obtain the data described previously, groups of boys in an institution were maintained on a diet which provided them each with approximately 3000 cal./day. This diet was rigidly controlled, could not be supplemented, and was, so far as could be arranged, the same both in quantity and quality for every boy. It did, however, vary from time to time as different foods became available. The vitamin C content of the diet was determined periodically throughout the year by the usual chemical means on representative samples of different foods taken at the time of serving. The vitamin P content of the diet was assessed by making double determinations of capillary resistance (C.R.) before and after a period of usually more than 20 days on the diet, by comparing the differences, factorized to a standard initial C.R. of log 2·48 (Scarborough & Wokes, 1946, 1947), with the hesperidin dose/response curve and assessing potency in provisional units (P.U.) per day on the basis of 1 g.

hesperidin ≡ 100 mg. W.S.P.I. ≡ 100 P.U. (Bacharach & Coates, 1942). Seasonal variations in intake of both vitamins were found, that of vitamin C increasing from a minimum of 23 mg./day in May–June to a maximum of 50–55 mg./day in October–November, an increase which was reflected in alteration in the mean plasma ascorbic acid concentrations from 0·18 mg./100 ml. in May to 0·80 mg./100 ml. in November. The vitamin P intake increased gradually from 7 P.U./day in February–March to 15 P.U./day in August–September and fell thereafter. Such a diet, while probably fulfilling the vitamin C requirements during the late summer and autumn, provides, even at its best, much less than the daily requirement of vitamin P, which is not less than 33 P.U./day and may be considerably more.

The ratio between the vitamin C and vitamin P contents of the diet as eaten (measured as mg. and P.U. per 100 g. respectively) ranged from 1·7 to 5·5. This ratio for uncooked potatoes and cabbage was found by Bacharach & Coates (1943) to be only 0·5. This discrepancy supports the view that the loss of vitamin P during cooking is relatively much greater than that of vitamin C.

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The Distribution of Nucleic Acids in Bone Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (*From the Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1, *and the Department of Pathology, The British Postgraduate Medical School, London, W.* 12)

Red marrow obtained by sternal puncture from normal and diseased individuals has been examined by the histochemical test of Brachet (1942), using purified ribonuclease from ox pancreas. Basophilic material, with strong affinity for basic dyes such as pyronin, is distributed in greatest amount in the cytoplasm and nucleoli of the youngest free cells; diminution of basophilia occurs with maturation, and is less in all stages in granulocytic than erythrocytic series. The basophilic material is removable by ribonuclease, and is interpreted as containing ribonucleic acid.

In the hyperplastic marrow of conditions such as pernicious, chronic haemolytic or severe iron-deficiency anaemias, the earliest cell types, particularly of the erythrocytic series, are increased; general cellularity is increased, with fewer fat cells. The ribonuclease test suggests an increase in ribonucleic acid in such marrows, concentrated largely in the cytoplasm and nucleoli of the earlier cells (White, 1947).

An attempt has been made to substantiate the findings in sternal marrow by chemical analysis for ribo- and deoxyribonucleic acids, as well as by histological examination. The samples usually weigh between 30 and 60 mg. and the nucleic acids are estimated after the method of Schmidt & Tannhauser (1945), nucleic acid phosphorus being determined by the ultra-micro method of Berenblum & Chain (1938), as modified by Davidson & Waymouth (1943).

Estimations on the sternal marrow of ten normal individuals yield the figures shown in the Table, expressed as milligrams of phosphorus per 100 g. of fresh tissue.

Results for deoxyribonucleic acid are the least satisfactory since with the quantity of tissue available the amount of phosphorus to be estimated is extremely small. A fairly wide variation in the cell population is found in normal marrow.

Composition of aspirated sternal marrow from ten normal individuals mg./100 g.

	Total nucleic acid phosphorus	Ribonucleic acid phosphorus	Deoxyribonucleic acid phosphorus
Mean	20·7	14·2	6·9
Standard deviation	5·9	3·6	3·5
Observed range	13·0–29·0	10·3–18·3	2·7–13·0

In many blood dyscrasias, particularly severe anaemias, an increase in both nucleic acids is found (often exceeding 3 S.D.), and the histological picture indicates a considerable hyperplasia, with particular increase in the earlier cell types. Three cases of pernicious anaemia, and two macrocytic anaemias of different aetiology have also been followed throughout therapy. A fall in marrow nucleic acids has occurred during the reticulocyte response, the cellularity still being great, but the degree of maturity considerably more advanced. As the blood picture improves, the marrow gradually returns to a normal histology and cellular distribution, and the nucleic acid levels fall to within normal limits.

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A Simple Medium for Production of Antibiotic by *Bacillus licheniformis*. By P. D'Arcy Hart (*National Institute for Medical Research, Mill Hill, N.W.* 7) and G. M. Hills (*Microbiological Research Department, Porton*)

Callow & Hart (1946) described the production of antibiotic material by a strain of *Bacillus licheniformis*; the material was active against Mycobacteria and it has since been provisionally named 'licheniformin'. Whilst a suitable synthetic medium was desirable in order to simplify the isolation of the active material, production on the medium used was limited by supplies of asparagine, and the use of glucose in it had other disadvantages, e.g. a critical optimum concentration and production of troublesome by-products.

The first modification arose from the observations (*a*) that the maximum titre was not reached until about 4 days after the initial acid fermentation had ceased, and the medium had regained neutrality or had become alkaline, and (*b*) that the maximum titre was reached in less than 2 days in modifications of the CCY medium of Gladstone & Fildes (1940), which never became acid. Replacement of glucose by lactate up to 1% gave a high titre in about 2 days; the titre was little affected by variation of sodium lactate concentration between 0·2 and 0·8%.

In order to replace the asparagine by a simpler source of nitrogen, modifications of the medium of Fildes (1938) were tested. These contained sodium lactate and NH_4^+ salts and gave good growth, but little antibiotic. The nitrogen content of the medium was, however, about a quarter of that of the asparagine medium. A 2- to 4-fold increase in the concentrations of NH_4^+ and lactate gave a titre of 160 units/ml., though further increase was inhibitory. It was thus possible to dispense with both the asparagine and the glucose of the medium of Callow & Hart.

In a further simplified medium, which actually gave higher titres, the NH_4^+ salts were replaced by NH_4^+ lactate, the amount of sodium lactate being correspondingly reduced. Satisfactory concentrations were sodium lactate (50%) and NH_4^+ lactate (syrupy), each 10 ml./l., with other salts as used by Callow & Hart. The fluid medium was inoculated with about 10^8 spores/l. and incubated for 6 days in layers of 10 mm. average depth in enamelled trays with lids. Under these conditions batches of about 200 l. regularly give titres of 400–600 units/ml., the final pH of the culture being 8·8–9·6. The active material is then largely free in the medium.

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Licheniformin, the Antibiotic Material from *Bacillus licheniformis*; Concentration and some Chemical and Biological Properties. By R. K. Callow, R. E. Glover and P. D'Arcy Hart. (*National Institute for Medical Research, Hampstead and Mill Hill, London*)

Substantial improvements have now been made on the method previously described (Callow & Hart, 1946) of concentrating this antibiotic material. Cultures obtained by the method of Hart & Hills (1946) were brought to pH 2·5 and boiled or autoclaved (10 lb./in.^{2} pressure) for 10 min. The solid was removed and the fluid, at pH 5, run through a bed of activated charcoal (5–7 g./l.) mixed with kieselguhr. Active material was eluted from the washed charcoal with a mixture of water, *n*-butanol and conc. HCl (89 : 10 : 1), and the eluate was evaporated to small bulk under reduced pressure. The acid solution was run through a column of charcoal. The filtrate was brought to pH 10, an inactive precipitate removed, and then neutralized. Excess of picric acid solution was added and the precipitate decomposed by dilute

HCl, the picric acid extracted by ether, and the aqueous solution poured into acetone. The helianthate or reineckate could also be used for separation. The amorphous material, precipitated by acetone, behaved as the hydrochloride of a base, equiv. wt. 320; the sulphate was similar. The material gave the Sakaguchi reaction but not the reaction for glucosamine (Elson & Morgan, 1933) or the modification given by streptomycin (Scudi, Boxer & Jelinek, 1946). The helianthate when recrystallized gave material of unchanged antibiotic activity; it yielded a hydrochloride which had $[\alpha]_D - 37°$ in water and contained C, 40·0; H, 7·6; N, 17·3; Cl, 9·8; amino-N, 2·6%. It is now proposed to name the active base *licheniformin*. It appears to differ from other antibiotic materials reported from aerobic spore-forming bacilli. Of a number of strains of this group examined, only those which were identifiable as *B. licheniformis* (Gibson, 1944, 1946) yielded licheniformin-like activity under the conditions of culture used.

Batches of material prepared as described were of approximately constant activity against *Mycobacterium phlei*, inhibiting surface growth in broth at a dilution of 1 in $2·5–5·0 \times 10^6$. The growth of three virulent strains of *M. tuberculosis*, after deep culture for 2 weeks in a Dubos-type synthetic medium (inoculum concentration 0·007 mg. (moist weight)/ml.), was also inhibited at dilutions ranging from 1 : 160,000 to 1 : 640,000. High or moderate bacteriostatic action was shown, further, against a number of strains of *Staph. pyogenes*, *Corynebact. diphtheriae*, *B. anthracis*, *Str. pyogenes* and Pasteurellae. *E. coli* was distinctly less sensitive.

Mice of 20 g. injected i.v., i.p., or s.c. with 5 mg. of the material survived, whilst injections of 10 mg. i.v. were usually fatal within 1–2 hr. and i.p. and s.c. within 24 hr. Mice experimentally infected with a licheniformin-sensitive *Staph. pyogenes*, and treated with the antibiotic material, were partially protected (Mrs J. Bedford). Greater protection was afforded in a *B. anthracis* infection, the fatality rate among 40 treated mice being 16% as compared with 95% among 40 controls (Dr J. M. Barnes). Mice infected by the respiratory route (Glover, 1944) with bovine-type *M. tuberculosis*, and treated for about 40 days with 6 mg. daily in divided doses, showed some suppression of the pulmonary lesions similar in extent to that in streptomycin-treated mice: this effect needs confirmation.

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A Comparison between Single-sample Tests for Liver Damage. By C. A. Mawson. (*Pathological Laboratory, Royal Berkshire Hospital, Reading*)

If several tests are to be done it is desirable that only one blood sample should be taken, and the tests selected for investigation were the Takata-Ara (Britton, 1944), thymol turbidity (Maclagan, 1944*a*), colloidal gold (Maclagan, 1944*b*), alkaline phosphatase (King, Haslewood & Delory, 1937), Van den Bergh (Haslewood & King, 1937) and albumin-globulin ratio (Robinson & Hogden, 1940). These tests have been applied to 140 pathological sera, including 25 cases of obstructive jaundice proved at operation and 33 cases of infective hepatitis diagnosed on clinical grounds. The three flocculation tests have also been done on sera of 100 apparently normal blood donors.

Normals. The degree of flocculation in each of the three tubes of the Takata-Ara reaction was expressed numerically as follows:

$+ = 3$, $\pm = 2$, trace $= 1$, no precipitate $= 0$.

The thymol turbidity tests were read in a photoelectric colorimeter. The results on 100 normal donors are given in Table 1.

Table 1

Test	Mean	Standard deviation	Mean ± 2 × S.D.
Takata-Ara	0·41	0·922	0–2·2
Colloidal gold	0·34	0·808	0–1·9
Thymol turbidity	1·21	0·527	0·1–2·3

Obstructive jaundice and infective hepatitis. Table 2 gives a comparison which shows that the difference between the results of the Takata-Ara reaction in these two conditions was barely significant. The colloidal gold, thymol turbidity and phosphatase tests were very significantly different, but no significant difference was found with the proteins or Van den Bergh reaction.

Table 2

	TA (units)	CG (units)	TT (units)	TP (%)	A (%)	G (%)	A/G	Ph (units/100 ml.)	Bil (mg./100 ml.)
Infective hepatitis:									
Mean	4·77	3·16	5·12	6·51	3·23	3·29	1·07	16·18	8·32
S.D.	2·04	1·70	2·62	1·00	0·95	0·92	0·39	6·59	6·54
n	31	31	31	28	28	28	28	32	30
Obstructive jaundice:									
Mean	2·48	0·12	0·75	6·18	3·10	3·06	1·05	37·30	14·45
S.D.	2·22	0·33	0·54	0·66	0·56	0·55	0·29	15·24	8·58
n	25	25	25	25	25	25	25	25	25
Difference of means / Standard error	1·98	4·88	4·52	0·71	0·31	0·51	0·11	3·24	1·47

TA = Takata-Ara; CG = colloidal gold; TT = thymol turbidity; TP = total proteins; A = albumin; G = globulin; Ph = phosphatase; Bil = bilirubin.

Considering results on individual patients, there was no differentiation in the case of the Takata-Ara, proteins or Van den Bergh reaction. Phosphatase values were higher in obstructive jaundice than in hepatitis (Maclagan, 1944*c*). Only four results lower than 25 units were observed in the former condition, while four cases of infective hepatitis gave results higher than 25 units. The distinction was clearer with the colloidal gold test. In obstruction no result higher than grade 1 was observed, but five cases of infective hepatitis gave results of 0 or 1. The thymol test in obstructive jaundice was always 2·1 units or less, but in infective hepatitis five results of 2·1 units or less were obtained. No case of obstructive jaundice would have been incorrectly diagnosed by these tests, but five cases diagnosed clinically as infective hepatitis gave results which could not have distinguished them from obstructive jaundice.

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Metabolism of Naphthalene. By J. Booth and E. Boyland. (*Chester Beatty Research Institute, Royal Cancer Hospital (Free), London, S.W. 3*)

Anthracene is metabolized in part to give a diol, 1:2-dihydroxy-1:2-dihydroanthracene (Boyland & Levi, 1935), and 3:4-benzpyrene may be metabolized in part though an analogous diol (Weigert & Mottram, 1946). These diols are easily dehydrated to give phenols, and it is conceivable that the phenolic products isolated as metabolism products of other polycyclic hydrocarbons (e.g. Berenblum & Schoental, 1946) arise through the intermediate formation of α:β-diols by the reaction which has been called perhydroxylation because it involves the addition of the elements of hydrogen peroxide. Until recently such diols had only been actually isolated in anthracene metabolism, but they have now been obtained as products of naphthalene metabolism. Young (1946) describes a 1:2-dihydroxy-1:2-dihydronaphthalene as excreted by rats treated with naphthalene. This dihydroxydihydronaphthalene melts at 127–128° and is optically active ($\alpha_D = -154°$). We had simultaneously isolated a 1:2-dihydroxy-1:2-dihydronaphthalene from the urine of rabbits dosed with naphthalene. This rabbit naphthalene diol is optically inert and melts at 98°. The experiments of Young and of ourselves show that the conversion of an aromatic hydrocarbon into a diol in the animal body occurs with naphthalene in addition to anthracene, so that it may be a general reaction for compounds of this type. The 1:2-dihydroxy-1:2-dihydroanthracene produced by rabbits is apparently a racemic mixture (Boyland & Shoppee, 1947), and the rabbit naphthalene metabolite also appears to be a racemate. It is remarkable that a racemate should be produced in a biological process, but the possibilities of racemization in the urine are being investigated.

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The Estimation of Miracil in Urine. By R. V. Coxon, A. L. Latner and E. J. King. (*British Postgraduate Medical School, London*)

Miracil (1-methyl-4-diethyl-β-aminoethylaminothioxanthone) has been introduced for the treatment of schistosomiasis. It can be estimated in urine by a colorimetric technique similar to that described for mepacrine by King & Gilchrist (1945), in amounts from 0·5 mg./l. upwards. The drug is extracted from alkaline urine, into ether, which is washed with NaOH; it is then taken into N-HCl and the yellow colour measured.

To 50 ml. of urine in a separating funnel is added a sufficient volume (2–3 ml.) of 0·3N-NaOH to render the mixture alkaline to litmus and also 50 ml. of diethyl ether; the funnel is vigorously shaken. After settling, the lower layer is collected in a flask and the upper transferred to a stoppered bottle. The lower layer (which is likely to contain some emulsion) is now returned to the funnel and shaken with a further 50 ml. of ether. On separation, the upper layer is added to the ether extract obtained from the first operation, while the lower is again returned to the funnel and mixed with about 100 ml. of water. This tends to break up any remaining emulsion enabling a further amount of ether extract to be recovered. The whole of the ether extract is now washed with 10 ml. of 0·3N-NaOH, any emulsification being counteracted with a few drops of absolute ethanol. The ether layer, after a further washing with water, is then shaken with exactly 10 ml. of N-HCl. The acid layer is withdrawn and its colour compared with standards made up by dissolving 5 and 10 mg. respectively of miracil in 1 l. of N-HCl.

If the reading indicates a urinary content of less than 0·5 mg./l. of miracil, it is preferable to repeat the procedure using 100 ml., or even 200 ml., of urine.

Miracil can be identified by means of the ruby colour which it gives when treated in chloroform solution with acetic anhydride and concentrated sulphuric acid. Urine from a rabbit injected with miracil contains in addition to miracil, identifiable by this test, other yellow material which can be extracted into ether but which cannot be transferred therefrom to HCl.

The concentration of the drug in rabbit urine following a dose of 25 mg./kg. body weight was about 2·5 mg./l.

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Acid-soluble P Compounds in Bone Marrow. By C. Lutwak-Mann. (*School of Biochemistry, Cambridge*)

Trichloroacetic acid extracts of fresh bone marrow (rabbit) contained per 100 g. wet weight, 25–30 mg. total acid-soluble P, of which 3–5 mg. was inorganic P, 7–11 mg. hydrolyzable after 7 min., 1–2 mg. between 7 and 30 min., 0·5–1·0 mg. between 30 and 120 min., in N-HCl at 100°. Of the phosphate hydrolyzable in 7 min., only part, sometimes only half, could be accounted for by ATP. Values for ATP determined by Ba precipitation and enzymic deamination (Parnas & Lutwak-Mann, 1935) were 0·8–1·5 mg.% adenine-NH_2-N (corresponding to 3·6–6·6 mg.% pyro-P). The level of ATP was not affected by preceding consecutive bleedings of the animals. In addition to ATP, rabbit bone marrow contained in the Hg-precipitable nucleotide fraction some material which did not form an insoluble Ba salt at pH 8·3, but which when acted upon by muscle deaminase yielded 1–1·5 mg.% NH_3-N.

When a suspension of bone marrow in phosphate-free Ringer solution was incubated at 20° for 2–4 hr., there was considerable increase in inorganic P, especially anaerobically; M/50-NaF did not prevent the rise of inorganic P. In oxygen, readily hydrolyzable P esters chiefly contributed to the accumulation of inorganic P, whereas anaerobically it was

derived mainly from organic P which resisted 120 min. acid hydrolysis. Total acid-soluble P remained stable during incubation.

It was, however, interesting to note that even after prolonged incubation (in O_2 or N_2) half or more of the P_7-P_0 fraction still remained. Simultaneous analysis of ATP and free NH_3 showed that the ATP content was little affected aerobically, but fell to about one-third anaerobically; NH_3 rose gradually in the course of 1–2 hr., both in presence and absence of O_2, from the initial value 0·8–1·5 mg. % to 3–4 mg. %, and did not increase further after longer incubation. Unlike in blood, the production of NH_3 in bone marrow was not prevented by borate. Rabbit bone marrow was found capable of deaminating adenosine (very rapidly), ATP, ADP, adenylic acid, adenine nucleotide, but not adenine, guanine or guanosine. It also contained phosphatases which acted vigorously upon metaphosphate, pyrophosphate, inosinate, adenylate, adenosinedi- and triphosphate, phosphoglycerate, fructosediphosphate, the esters of Cori, Neuberg and Embden.

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The Estimation of Urethane (Ethylcarbamate) in Blood. By H. E. Archer, L. Chapman, E. Rhoden and F. L. Warren. (*Chester Beatty Research Institute, Royal Cancer Hospital (Free), London, S.W. 3 and Pathology Department, Wellhouse Hospital, Barnet*)

In connexion with the recent use of urethane in the treatment of leukaemia (Haddow & Sexton, 1946; Paterson, Ap. Thomas, Haddow & Watkinson, 1946), the necessity arose for a method for estimating this substance in blood.

The very simplicity of the chemical structure, $NH_2.COOC_2H_5$, and its close resemblance to urea, indicated that attempts to estimate the substance by reactions based on the amino group were unlikely to be successful. Attention was therefore directed to the characteristic ester grouping. It was found that urethane is quantitatively hydrolyzed to alcohol, ammonia, and carbonate, by boiling with NaOH solution. The alcohol can be estimated by distillation into acid potassium dichromate solution and titration of excess dichromate with sodium thiosulphate after addition of potassium iodide.

The estimation involves two stages.

(1) *Determination of volatile reducing substances in blood before alkaline hydrolysis.* 10 ml. of Folin-Wu blood filtrate is measured into a 100 ml. flask containing a few glass beads and connected with a Jackson's condenser which has its delivery tube extended downwards for about 15 cm. The delivery tube dips below the surface of a mixture of potassium dichromate and sulphuric acid (1 ml. of 0·1 N-potassium dichromate and 5 ml. Analar conc. sulphuric acid) which is contained in a tube of 20 ml. capacity. The tube containing the mixture is well cooled in ice. With no water cooling on the condenser about 8 ml. of the flask's contents is distilled over into the oxidizing mixture. A glass stopper is sealed into the tube with a drop of sulphuric acid and the contents mixed and heated to 80° C. for 20 min. The tube is then cooled and the contents washed into a flask with about 100 ml. of water, 5 ml. of 5 % potassium iodide solution is added and the liberated iodine titrated with 0·1 N-sodium thiosulphate.

The above determination usually yields a small blank value.

(2) *Determination of volatile reducing substances in blood after alkaline hydrolysis.* A clean flask is fitted to the condenser and 10 ml. of blood filtrate together with 5 ml. of 40 % sodium hydroxide. The mixture is refluxed for 15 min. The water supply to the condenser is then stopped and 8–10 ml. of the flask's contents distilled into acid dichromate mixture as in (1). The subsequent procedure is as described in (1).

The difference between the dichromate reduced in (1) and (2) is a measure of the urethane content of the blood. 1 ml. of 0·1 N-dichromate is equivalent to 2·225 mg. of urethane.

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Inhibition of Acid Phosphatases by Formaldehyde. By M. A. M. Abul-Fadl and E. J. King. (*British Postgraduate Medical School, London, W.* 12)

The tissues and fluids of the body contain several phosphatases active at an acid pH (Roche, 1931; Kutscher & Wolbergs, 1935; Roche, Thoai & Baudoin, 1942; Behrendt, 1943). These are characterized by differences of pH optimum, of rate of hydrolysis of different substrates, and of the effects of various activators and inhibitors. Herbert (1944, 1946) differentiated prostatic phosphatase from that of normal serum by the ready destructibility of the former with ethanol. King, Wood & Delory (1945) found the acid phosphatases of prostate and red cells to be similar in many respects, including easy destruction by ethanol. Red blood cells contain roughly 100 times as much acid phosphatase as is present in serum, and haemolysis is, therefore, a potent source of error in the estimation of serum acid phosphatase.

A systematic study of the acid phosphatases of human tissues with several inhibitors has shown that those of the adrenal, intestine, liver, pancreas, spleen and thyroid are not affected by ethanol, whereas those of the bile, kidney, red cells and prostate are seriously inhibited. Formaldehyde treatment gives a sharp differentiation between the prostatic and red cell acid phosphatases. The latter is completely destroyed by the inclusion of 0·1 ml. of 20% neutral formaldehyde in the buffer-phenylphosphate-serum mixture used for the determination; while the prostatic phosphatase is quite unaffected. Other tissue acid phosphatases, including that of normal serum, are variably inhibited by formaldehyde, but none is so sensitive as the red cell enzyme. A high acid phosphatase after formaldehyde treatment strongly suggests a prostatic origin. This can be confirmed by the alcohol incubation method.

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DEMONSTRATIONS

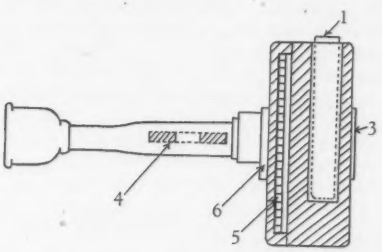
The Histological Appearances of Bone Marrow in Relation to the Nucleic Acids. By J. N. Davidson, I. Leslie and J. C. White. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1, *and Department of Pathology, British Postgraduate Medical School, London, W.* 12)

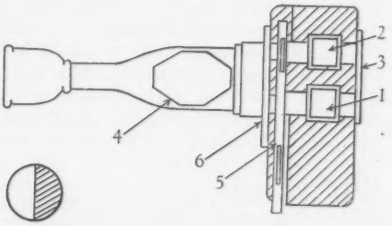
A Grey Wedge Photometer for Colorimetric Analyses. By E. J. King. (*British Postgraduate Medical School, London, W.* 12)

King & Delory (1944) described a photometer for field use which was made from a Lovibond comparator whose coloured disks were replaced by nine grey screens of density 0·1–1·0, and whose two fields (grey and coloured) were viewed through a complementary light-filter. In the photometer to be described the accuracy and ease of colour measurement is greatly increased by using a continuous grey-wedge in place of the disk of grey screens, and by bringing the two photometric fields together with an eyepiece. The construction is shown in the diagram. The solution to be examined is placed in the glass cell (1) alongside a similar cell (2) containing the solvent only, which serves to compensate losses due to absorption and reflexion. Both cells are illuminated by daylight or bright diffused lamplight falling on the diffusing screen (3). The two beams of light passing through the cells are brought together into the eyepiece by means of the Hufner rhomb (4). The field of view consists of two juxtaposed semicircular patches, each illuminated by the light from one cell only, with no dividing line between them.

Between the control cell (2) and the eyepiece is a continuously variable neutral wedge (5) (Ilford, Ltd.), consisting of a circular strip of gelatine, loaded with carbon or silver of uniformly increasing thickness enclosed between two glass disks, and increasing approximately uniformly with the angle

of rotation of the disk (for greatest accuracy, each wedge should be individually calibrated). A scale graduated directly in optical densities is attached to the periphery of the wedge holder. Interchangeable coloured filters, chosen to suit the particular solution being tested, can be dropped into the slot (6), so that both beams of light must pass through it.





To use the instrument, the cells are placed in position, a suitable filter inserted, the eyepiece sharply focused on the front edge of the Hufner rhomb, and the whole held up to the daylight. The neutral wedge is then rotated until the intensities of both halves of the field of view match. The optical density of the solution can be read off directly on the same scale.

This type of photometer can be used for a wide range of chemical estimations, if suitable filters are chosen (e.g. Ilford spectrum red for blue solutions, spectrum green for red or brown solutions, and spectrum violet (41/45) for yellow). It is robust, simple to use and quick in action. Its accuracy is comparable with that of the best Duboscq colorimeters and visual photometers.

I am greatly indebted to Mr L. G. Grimmett for his invaluable help with the construction of several models of the photometer.

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Cyan-haematin Method for the Determination of Haemoglobin. By E. J. King. (*British Postgraduate Medical School, London, W.* 12)

The colour produced by dissolving crystalline haemin in sodium cyanide solution is almost identical (by spectrophotometric measurements) with that produced by treating haemoglobin first with hydrochloric acid to decompose the haemoglobin and form acid haematin, and secondly with excess of sodium cyanide to transform the acid haematin into cyanhaematin. The colours are read with a Chance green or an Ilford tri-colour green filter, and are assessable on a stoichiometric basis, i.e. 4 mol. of haemin yield the same intensity of colour as 1 mol. of haemoglobin; or 1 mg. of haemin iron gives the same colour as 1 mg. of haemoglobin iron.

Standard. A solution of 28·8 mg. of pure haemin (8·57 % Fe) in 1 l. of 1 % sodium cyanide gives the same colour as 14·8 g. of haemoglobin (N.P.L. Haldane 100 % normal; cf. King, Gilchrist & Matheson, 1943) treated first with hydrochloric acid and then with excess sodium cyanide, and at a final dilution of 1 in 200. Samples of haemin of other than theoretical iron content should be used proportionately, e.g. 30 mg. of haemin of 8·2 % Fe $\left(\frac{28{\cdot}8 \times 8{\cdot}57}{8{\cdot}2} = 30\right)$.

Test. 0·5 ml. of blood are treated in a 100 ml. flask with 75 ml. N/10-HCl and left till the transformation to acid haematin appears to be complete, e.g. 5–10 min. Thereupon 20 ml. of 5 % NaCN solution are added and water to the mark.

Micro method. 0·05 ml. blood are added to 4·95 ml. N/10-HCl, left for 5–10 min., and then 5 ml. of 2 % NaCN added.

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Continuous Extraction Apparatus. By I. D. P. Wootton (introduced by E. J. King). (*British Postgraduate Medical School, London, W.* 12)

In the course of an investigation, it was necessary to extract a number of inorganic tissue residues with cold dilute acid until all soluble constituents had been leached out. Repeated extractions by hand were laborious and time-consuming; continuous extractors of the Soxhlet type provided difficulties in temperature and pH control. The extractor now in use houses the residue in a perforated crucible on a funnel, which drains into a large (2–5 l.) aspirator bottle. The bottle is filled with the extracting fluid, which flows in glass tubing forming a circuit from the bottom hole in the bottle to a point from which it runs on to the residue, and then back to the bottle. A continuous circulation is achieved by injecting compressed air at a suitable point in the circuit, thereby causing a hydrostatic inbalance.

An apparatus of the same design may be used with other solvents, provided they are not too volatile. Moreover, the extracting fluid may be held throughout at any temperature below its boiling point by immersion of the whole apparatus in a suitable bath. Thus, if necessary, the extraction may be carried out at a low temperature.

Estimation of Miracil in Biological Fluids. By A. L. Latner, R. V. Coxon and E. J. King. (*British Postgraduate Medical School, Ducane Road, London, W.* 12)

The **256th Meeting** *of the Biochemical Society was held at University College Hospital Medical School London, W.C.* 1, *on Saturday,* 15 *February,* 1947, *and took the form of a Symposium on 'The Relation of Optical Form to Biological Activity in the Amino-acid Series.'*

COMMUNICATIONS

Introductory Talk on Nomenclature of Amino-acids. By A. C. Chibnall. (*School of Biochemistry, Cambridge*)

The early literature and such reference works as Abderhalden's *Handlexicon* and Beilstein, name the amino-acids with a prefix that shows the direction of rotation of an aqueous solution of the substance in the neutral range. Thus, for the stereoisomers from proteins we have, for example, *d*-alanine, *l*-leucine. Subsequently the suggestion of Wohl and Freudenberg was widely adopted, according to which a prefix was used to denote the configurational family to which the alpha carbon atom of the amino-acid belongs, the actual direction of rotation being indicated by a plus or a minus sign enclosed in parentheses. Thus, for the same substances, we have *l*(+)-alanine, *l*(−)-leucine. This practice, however, is often simplified, and the protein amino-acids are referred to simply as *l*-amino-acids and their enantiomers as *d*-amino-acids. The above-mentioned substances thus appear as *l*-alanine and *l*-leucine, notwithstanding the fact that the first is in direct conflict with the early literature. Other difficulties are met with in practice, particularly in the case of threonine. The Editorial Boards of relevant Journals in the U.S.A. and this country have been concerned by the possibilities for confusion inherent in these diverse systems of nomenclature, and are hoping to agree on proposals for reform. The main points of interest will be outlined at the meeting.

The *d*- and *l*-Amino-acid Oxidases. By H. A. Krebs. (*Department of Biochemistry, The University of Sheffield*)

The subject will be dealt with under the following headings: History of the discovery of amino-acid oxidases. Evidence showing the existence of two types of amino-acid oxidases dealing with the members of the *d*- and *l*-series respectively. Properties of *d*- and *l*-amino-acid oxidases (chemical nature; specificity; distribution in different tissues and different species including micro-organisms; number of enzymes within each series; physiological significance of the *d*-enzyme; use of the *d*-enzyme for analytical purposes). Relations between transamination and amino-acid oxidases.

The Role of *d*-Amino-acids in the Nutrition and Metabolism of Mammals. By A. NEUBERGER. (*National Institute for Medical Research, Hampstead, N.W.* 3)

A selective breakdown of *l*-amino-acids by moulds was discovered by Schulze in 1886, but similar experiments with animals were not carried out until Wohlgemuth showed in 1905 that rabbits given racemic tyrosine, leucine, aspartic and glutamic acids, excreted the *d*-forms in the urine. These results have been extended to most naturally occurring amino-acids and to many other species. It appears that the organism is limited in its capacity to deal with both optical forms, but the threshold level at which excretion in the urine occurs, is considerably lower for the *d*- than for the *l*-isomeride. In most cases the excess *d*-amino-acid is excreted in the urine as such; however with *d*-glutamic acid ring closure to *d*-pyrrolidone-carboxylic acid occurs and with *d*-phenylalanine the product consists mainly of phenylpyruvic acid.

The replacement of an essential *l*-amino-acid in the diet by the *d*-isomeride and the resulting effect on growth has provided another approach to this problem. It appears that with methionine the two isomerides are fully interchangeable and this also probably applies to homocystine. With histidine, phenylalanine and tryptophan the *d*-amino-acid supports growth, but not as effectively as the corresponding *l*-isomeride, whilst *d*-leucine, *d*-isoleucine, *d*-valine, *d*-threonine and *d*-cystine are not available for growth in the rat. Interesting effects of configurational changes on metabolism have been found by du Vigneaud with *meso*-cystine, lanthionine and cystathione.

An actual conversion to the *l*-amino-acid in the body has been demonstrated for γ-phenyl α-aminobutyric acid, β-cyclohexyl-alanine, histidine and leucine. In all but one of these examples the method used consisted of labelling the carbon-chain of the *d*-amino-acid by deuterium. The acetylation which occurs with some of these compounds which are foreign to the body, has also been observed with S-aryl derivatives of cysteine. If such cysteine compounds have *d*-configuration, inversion usually precedes acetylation. In some cases, however, direct acetylation of brominated S-benzyl or S-phenyl *d*-cysteines has been observed.

The influence of optical isomerism on other biological reactions of amino-acids has been investigated, of which the following will be discussed: the formation of ketone bodies, the deposition of glycogen, the excretion of sugar in phlorrhizin-poisoned animals, the formation of histamine and urocanic acid, the excretion of homogentisic acid and phenylpyruvic acid in alcaptonuria and phenylketonuria respectively, and the transformation of tryptophan to kynurenic acid.

One of the most difficult tasks in this field is the correlation of the results obtained on the whole animal and the observations made on isolated tissues or enzymes. The great activity of the *d*-amino-acid oxidase on most *d*-amino-acids *in vitro*, is in striking contrast to the slowness with which these compounds are metabolized by the whole animal. It appears that the speed of intestinal absorption does not depend on optical isomerism and the scanty data available do not suggest that the rate of renal reabsorption differs for *l*- and *d*-amino-acids. It is possible that *d*-amino-acids penetrate less readily into other cells, such as those of the liver, or that the *d*-amino-acid oxidase is less active *in vivo* than would appear from *in vitro* experiments.

The Origin of *d*-Amino-acids in Proteins with Special Reference to *d*-Glutamic Acid. By G. R. TRISTRAM (read by A. C. CHIBNALL). (*School of Biochemistry, Cambridge*)

Interest was first aroused in the occurrence of *d*-amino-acids in protein hydrolysates by Kögl (1939) who isolated *d*-glutamic acid from hydrolysates of malignant tissue and claimed that the presence of *d*-amino-acids, and in particular *d*-glutamic acid, was peculiar to the proteins of malignant tissue. Within a year many other workers had shown that, although malignant tissues did yield small amounts of *d*-glutamic acid, the interpretation of Kögl was erroneous since small amounts of *d*-glutamic acid were also found in hydrolysates of proteins from normal tissues and of certain crystalline proteins.

The identification and estimation of *d*-amino-acids has (until recently) involved the isolation of the total acid so that the specific rotation can be determined. This method gives minimal values. Fortunately, new methods are now coming into use among which are isotope dilution, microbiological assay and enzymic methods.

Evidence has now accrued which shows that a certain amount of racemization occurs when *l*-glutamic acid is heated in 20% HCl. The degree of racemization which occurs on the hydrolysis of peptides appears to depend upon the hydrolytic procedure. Martin & Synge (1945) have summarized

the present viewpoint: 'Where an amino-acid is isolated in a partially or completely racemized state, it seems preferable to regard this as having arisen from the optically active form by racemization during the hydrolysis or isolation.' Synge (1944) also suggested that this view must be held until partial hydrolysis products containing residues of the *d*-amino-acids have been isolated.

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d-Amino-acids in Microbiological Chemistry. By H. N. Rydon. (*Lister Institute, London, S.W.* 1)

In only a few cases have *d*-amino-acids been found to be produced by micro-organisms, invariably as components of extracellular metabolic products; the structure of those substances of this class devoid of antibiotic properties will be briefly discussed.

In contrast with the rarity of the occurrence of *d*-amino-acids in their metabolic products, a surprisingly large number of micro-organisms have been found to respond to *d*-amino-acids, which may either be used for growth or, less frequently, show an inhibitory action. Such cases have been collected and will be discussed, particularly in relation to the importance of phenomena of this type in studies of bacterial nutrition and in the development of methods for the microbiological assay of amino-acids.

Possible mechanisms for the utilization and synthesis of *d*-amino-acids by micro-organisms will be discussed.

***d*- and *l*-Amino-acids in Antibiotics.** By T. S. Work. (*National Institute for Medical Research Hampstead, N.W.* 3)

Several crystalline polypeptides isolated from natural sources have been shown to be effective inhibitors of bacterial growth; other polypeptides possess striking growth-promoting properties. From some of the antibiotic polypeptides amino-acids of the unnatural *d*-configuration have been isolated. Penicillin may be regarded also as a derivative of a *d*-amino-acid and when synthesized from *d*-penicillamine has the full antibiotic activity of the natural product. Penicillin synthesized from *l*-penicillamine is biologically inactive.

Amino-acids of the *d*-configuration are frequently unsuitable as essential metabolites for organisms which cannot synthesize their own requirements, but are not usually growth inhibitory. X-ray mutants of moulds may show specialized growth requirements for one amino-acid and are then susceptible to inhibition by related amino-acids of the *l*-configuration but not by the corresponding *d*-isomers. In view of the non-antibiotic action of the *d*-amino acids, the question arises as to whether or not the antibiotics possessing *d*-amino-acids are active by virtue of their optical configuration.

Pharmacological Properties of *l*- and *d*-Amino-acids and Derivatives. By F. Bergel. (*Roche Products Ltd., Welwyn Garden City, Herts.*)

The simple *l*- and *d*-amino-acids do not possess any striking pharmacodynamic characteristics, in spite or because of their great importance as metabolics.

Nevertheless, there are certain indications in literature as to limited physiological activities and to some differences between optical antipodes of some of these amino-acids.

The pharmacological effects are more pronounced with more complex substances, such as halogenated or polypeptide-like derivatives. It is not impossible that amino-acid-highpolymers with pharmacodynamic actions exist which show a dependence of physiological potency on the stereochemical specificity of their optically enantiomorphic units. As long as such substances cannot be produced synthetically with differences in their optical properties, however, it is difficult to answer this question fully.

Finally, as one point, on the fringe of the problem under discussion, certain stereoisomeric drugs, related directly or indirectly to amino-acids, but not necessarily possessing the same optically active centre of the parent substance, differ sometimes widely in their pharmacological activity.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **Annual General Meeting** *of the Biochemical Society was held at Guy's Hospital Medical School on Saturday,* 15 *March* 1947, *when the following papers were read:*

COMMUNICATIONS

The Plastein Question. By J. A. V. Butler, E. C. Dodds, D. M. P. Phillips and J. M. L. Stephen. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.*1)

The formation of so-called plasteins from peptic and other digests has been known for a considerable time. Plastein formation from egg albumen was investigated in some detail by Borsook & Wasteneys (1924), who considered that a resynthesis of peptide bonds had been shown to occur in peptic digests which had been concentrated and incubated with more pepsin at pH 4 and other values. The evidence was (1) the proportion of N precipitated by trichloracetic acid was increased by this treatment; (2) the precipitate had a lower proportion of amino-N than the original digest; (3) no action occurred when boiled pepsin was added. Similar observations were made with insulin by Fisher & Scott (1933) and Haddock & Thomas (1942).

We have found that when pepsin digests of insulin stand at various pH's, both more acid and more alkaline than the original, a precipitate is formed which may amount to 50% of the original insulin. Trichloracetic acid does not precipitate any additional material. The formation of the precipitate is accelerated by heating, but it also occurs in the cold, even when the digest is frozen. The same occurs when the pepsin has been inactivated by 5 min. heating to 60° at pH 7·8. The precipitate has a lower percentage of amino-N than the digest as a whole. The change of amino-N in the whole digest, however, is small, so that there is no evidence that the resynthesis of peptide bonds has occurred. The digest has merely been divided into two parts with greater and less amino-N content.

As the peptic digest ages, the amount of precipitate obtainable from it diminishes. The phenomenon appears to be similar to the denaturation of proteins, and it is suggested that the larger fragments of insulin produced by pepsin retain at first something of the original structure of the protein. On heating, such fragments undergo denaturation and become insoluble. The molecular weight of the insoluble substance, when redissolved, is of the order of 12,000, i.e. about the same as a submolecule of insulin. It must therefore either consist of a more or less complete submolecule or portions of more than one. It is complex in composition, the following eight amino-acids having been detected by paper chromatography: aspartic acid, cystine, tyrosine, glutamic, glycine, alanine, leucine and there are certainly others, as several spots were not identified. The cystine is approximately equally divided between the precipitate and the filtrate.

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The Thymol Flocculation Test. By N. F. Maclagan. (*Westminster Hospital Medical School, S.W.*1)

In the original description of the thymol turbidity test as an indicator of liver dysfunction it was noted that in the case of positively reacting sera the mixture usually flocculated on standing overnight (Maclagan, 1944*a*, 1944*b*). More recently Maclagan & Bunn (1947) have shown that only hepatitis gamma globulin fractions separated from hepatitis serum flocculate the thymol reagent (with added cephalin), normal gamma fractions producing turbidity without flocculation. Neefe (1946) and Neefe & Rheinhold (1946) have also made extensive clinical observations on this thymol flocculation test in hepatitis, and regard it as a valuable addition to the turbidity test. A search of past records of sera tested by the writer was therefore made, and yielded 819 cases in which parallel observations of turbidity and flocculation had been recorded. In 727 (89%) the two tests were in agreement, in 64

(8%) turbidity was positive (over 4 units) with negative flocculation, and in 27 (3%) turbidity was normal with positive flocculation. Analysis of the 91 cases where the two tests were in disagreement gave the results shown in the accompanying table, from which it appears that the turbidity test is on the whole a slightly more sensitive one, and has a particular advantage in certain types of jaundice such as that following administration of serum or arsenicals. The flocculation test, however, gives approximately a 90% agreement and has the advantage of not requiring standards for comparison, being graded simply as 0, 1, 2, 3 or 4+. The material available did not throw any light upon the claims of Neefe & Rheinhold (1946) that in hepatitis the flocculation test usually becomes positive sooner and remains positive longer than the turbidity test.

Diagnosis	Turbidity raised Flocculation negative (64)	Turbidity normal Flocculation positive (27)
Hepatitis	17	4
Obstructive jaundice	3	1
Haemolytic jaundice	0	2
Post arsphenamine jaundice	9	0
Homologous serum jaundice	2	0
T.N.T. jaundice	2	0
Leptospiral jaundice	4	0
Malaria	4	4
Arthritis	5	3
Miscellaneous	18	13

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Clinical Assay of Vitamin P. IV. A Method for Evaluating the Potency of Rose-hip Preparations. By H. Scarborough and F. Wokes. (*From the Clinical Laboratories of the Royal Infirmary, Edinburgh, and the Ovaltine Research Laboratories, King's Langley, Herts.*)

In a previous paper we described the evaluation of the vitamin P potency of the experimental diet against the hesperidin log dose/response curve. Such a procedure involves the assumption that the log dose/response curve for vitamin P from natural sources has a similar slope to that found for hesperidin. Evidence justifying this assumption is now presented by comparing the log dose/response curves for four different preparations of rose hips with that for hesperidin. The results of four point assays (which allow for any difference in the slopes of the log dose/response curves) on each of four preparations of rose hips have been compared with the figures obtained by evaluating separately at two different dosage levels against the hesperidin log dose/response curve. The coefficient of variation of a single assay was usually about 15 but might reach as high as 30.

There are two factors which affect the accuracy of such assays, viz. the daily dose of the preparation containing vitamin P and the time for which it is given.

(*a*) When the total dosage given over the 5-day test period was 50 P.U., the coefficient of variation of a group of five boys (V) ranged from 35 to 12·5; when the total 5-day dosage was 100 P.U., V lay between 12·3 and 3·4, and at a level of 150 P.U., $V = 8{\cdot}4$.

(*b*) At dosage levels of the order of 10–20 P.U./day there is an indication that V becomes less as the period over which the dosage is spread is extended. Thus in one series of assays V fell from an average of 13·2 to 3·6 as the period of dosage increased from 5 to 30 days.

For these reasons, it is recommended that in the clinical assay of vitamin P the dosage level should be at least 20 P.U./day given over a period of not less than 5 days.

Estimation of 'Pregnanediol-like Glucuronide' in Human Urine. By N. G. Bisset, B. W. L. Brooksbank and G. A. D. Haslewood. (*Guy's Hospital Medical School, S.E.*1)

Using solutions of 'sodium pregnanediol glucuronidate' (NaPG; Marrian & Gough, 1946) we have worked out an analytical method involving extraction with butanol, entrainment of the Ba-salt of the glucuronide on a Ba-phosphate precipitate and final estimation of the glucuronic acid by the naphthoresorcinol reaction. Factors for loss on butanol extraction and on entrainment have been established. About 0·1 mg. of NaPG can easily be entrained and estimated. Application of this method

to human urine reveals the presence in normal male and 'follicular' female urine (adequately preserved) of a fraction of glucuronide closely resembling NaPG in its solubilities (see also Allen & Viergiver, 1941; Westphal, 1944). We have called the total glucuronide found in this fraction *pregnanediol-like glucuronide* (*PLG*). As estimated by our method, PLG excretion is about 10–20 mg./day (as NaPG) in 'follicular' female urine. The amount isolated can be varied somewhat with the conditions of butanol extraction and entrainment. PLG output rises in the luteal phase of the female cycle and, of course, very greatly in pregnancy; its level in adrenal hypertrophy and in other conditions should be of interest. The chemical nature of PLG is being investigated.

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A Method of Estimating Peptic Activity in Gastric Contents. By J. N. Hunt (introduced by G. A. D. Haslewood). (*Guy's Hospital Medical School*)

The estimation of proteolytic activity is complicated by the difficulty of preparing a reproducible protein substrate (Northrop, 1932). This problem was partly solved by the haemoglobin method of Anson & Mirsky (1932). The substances soluble in trichloracetic acid and giving a blue colour with Folin and Ciocalteu's phenol reagent were used as a quantitative index of the proteolysis of a substrate of carboxyhaemoglobin.

The method to be described is a modification using a substrate of dried citrated plasma or serum. Samples of substrate obtained from widely differing sources have given constant results so that the tedium of preparing carboxyhaemoglobin is avoided.

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The Oxidation of Diiodotyrosine. I. 3:5-Diiodo-4-hydroxybenzaldehyde as a Possible Intermediate in Thyroxine Formation. By Rosalind Pitt Rivers. (*National Institute for Medical Research, Hampstead, N.W.3*)

During aerobic or peroxide oxidation of diiodotyrosine, diiodo-*p*-hydroxybenzaldehyde is produced as well as thyroxine. At the same time oxalic acid is formed in a molecular amount equivalent to the sum of thyroxine and the aldehyde. This suggests that both may be formed by oxidation of diiodotyrosine on the β-carbon atom of the side chain and leads to the hypothesis that the aldehyde might be an intermediate in the thyroxine synthesis, by further loss of the 1-carbon aldehyde residue.

Objections to this hypothesis are, firstly, that Dakin (1909) was unable to oxidize 3:5-diiodo-*p*-hydroxybenzaldehyde to the quinol with peroxide although he demonstrated the conversion of the dichloro- and dibromo-analogues to the corresponding quinols; secondly, that incubation of diiodotyrosine in the presence of the aldehyde fails to reveal any increase in the yield of thyroxine. Nevertheless, the possibility of complete loss of the side chain of diiodotyrosine with a resulting reactivity on the *para*-position to the phenolic group is indicated by the formation of 2:4:6-triiodophenol both from diiodotyrosine and the aldehyde during the drastic oxidation of these compounds. It may be that this more resistant aldehyde requires the presence of a catalyst such as a heavy metal (Cu or Fe) to permit oxidation of the side chain. This is being further investigated.

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Absorption of 3-Methyl Glucose from the Small Intestine of the Rat and Cat. By P. N. CAMPBELL and H. DAVSON. (*University College, London, W.C.*1)

With the isolated loop technique it was found that 3-methyl-glucose was absorbed from the small intestine of the rat 16% less rapidly than glucose, and therefore considerably more rapidly than fructose and the pentoses.

That 3-methyl-glucose is actively absorbed from the small intestine of the cat was shown by measuring its absorption when the blood concentration was maintained at a higher level than that in the gut. Under these conditions 30% of the added 3-methyl-glucose was absorbed in 30 min., whilst the residual 3-methyl-glucose concentration in the gut after this interval was approximately 60% of that in the blood.

These results throw some doubt on the 'phosphorylation' theory of intestinal absorption (Verzar & McDougall, 1936).

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A Precursor of the Dimethyl Sulphide evolved by *Polysiphonia fastigiata*. Dimethyl-β-propiothetine (Dimethyl-β-carboxyethylsulphonium Hydroxide) and its Salts. By FREDERICK CHALLENGER and MARGARET ISABEL SIMPSON. (*The University, Leeds*)

On exposure to air the marine algae *Polysiphonia fastigiata* and *P. nigrescens* evolve dimethyl sulphide (Haas, 1935). Assuming that the sulphide is eliminated from some more complex compound Dr Haas kindly allowed us to study the problem.

The alga, plucked from its host (*Ascophyllum nodosum*) was left in cold absolute alcohol for 2–3 weeks and the extract concentrated at about 40° under diminished pressure to a brownish-green syrup which yielded a brown solid on trituration with alcohol. This had an acid reaction and with cold sodium hydroxide evolved dimethylsulphide, characterized as the mercurichloride. It gave a precipitate with aqueous ammonium reineckate. From this the reineckate ion was removed by addition of silver sulphate in acetone, filtration and removal of sulphate by barium chloride (Kapfhammer & Bischoff, 1930). Evaporation of the filtrate at 40° gave a semi-solid residue which evolved dimethyl sulphide as before. In preliminary experiments this was converted to the platinichloride which, with metallic silver (Dudley, 1929), gave silver chloride, platinum and, on filtration and evaporation as before, a solid chloride ($(CH_3)_2S$ with NaOH) which was converted to the picrate. Later the platinichloride stage was omitted and the chloride converted directly to the picrate ($(CH_3)_2S$ with NaOH). Treatment of the picrate with hydrochloric acid, extraction with ether, and evaporation as before gave the chloride, m.p. on recrystallization from alcohol. This was optically inactive, evolved $(CH_3)_2S$ with NaOH and could be reconverted to the picrate. (Found: C, 35·7, 35·7; H, 6·6, 6·6; Cl, 20·3, 20·6; S, 18·9, 19·0; equivalent 172·0; $C_5H_{11}O_2ClS$ requires C, 35·3; H, 6·4; Cl, 20·7; S, 18·7; % equivalent 170·5.)

The acidity of the chloride and the absence of reaction with 2:4-dinitrophenylhydrazine excludes such constitutions as

(I) $(CH_3)_2\underset{\widetilde{Cl}}{S}.CH_2COCH_2OH$ (α-thetine chloride)

or (II) $(CH_3)_2\underset{\widetilde{Cl}}{S}.CHOHCH_2CHO$ (β-thetine chloride).

Two structural formulae appeared probable:

$$(CH_3)_2\underset{\widetilde{Cl}}{S}\text{–}CH(CH_3)COOH$$

and

$$(CH_3)_2\underset{\widetilde{Cl}}{S}.CH_2CH_2COOH.$$

The corresponding thetine bromides (Biilmann & Jensen, 1936; Carrara, 1893; Holmberg, 1946) were prepared and converted to the picrates and other salts with results as shown below:

	Chloride	Bromide	Picrate	Styphnate	Platinichloride
α-Thetine deriv. m.p. ° C.	—	94–95	150	151·5–152	111
β-Thetine deriv. m.p. ° C.	134	112–114	132	148	193 or 200*
Mixed m.p. ° C.	—	74	124	139	105–145
Algal product m.p. ° C.	134	111–113	132	148	193 or 200*
Algal product + α-thetine deriv. Mixed m.p. ° C.	—	75	122	138	105–145
Algal product + β-thetine deriv. Mixed m.p. ° C.	133–134	111–112	131–132	148	193 or 200*

* Dependent on rate of heating.

These results clearly indicate that the algal product is the chloride of dimethyl-β-propiothetine (II).

Only one other instance of the isolation of a sulphonium compound from a natural product is recorded. Neuberg & Grosser (1905–6) state that the precursor of the diethyl sulphide evolved on warming the urine of dogs with alkali is methyldiethyl-sulphonium hydroxide, which was isolated as a salt by successive precipitation with phosphotungstic acid and $KBiI_4$; experimental details are very scanty. Toennies (1940) and Toennies & Kolb (1945) have, however, suggested that sulphonium compounds, e.g. derivatives of methionine, may be found to play a part in biological processes.

Haagen-Smit, Kirchner, Deasy & Prater (1945) have isolated

$$CH_3SCH_2CH_2COOCH_3$$

from pineapple juice. This may have arisen by isomeric change of the thetine anhydride

$$(CH_3)_2S^+.CH_2CH_2COO^-$$

by a process analogous to the conversion of betaine to the methyl ester of dimethylaminoacetic acid.

$$(CH_3)_3N^+.CH_2.COO^- \rightarrow (CH_3)_2N.CH_2.COOCH_3$$

(Willstätter, 1902). Attempts to effect this change are in progress.

The sulphonium compound may occur in the alga as a more complex product, possibly containing a peptide link or as an ester containing a sugar or a sugar alcohol. Hydrolysis may have occurred during concentration of the alcoholic extract. This question is now being studied.

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Digitonides of Steroid Ketones. By G. A. D. Haslewood. (*Guy's Hospital Medical School, S.E.* 1)

'Insoluble' digitonides are given by certain steroids with a keto-group at C_3. For instance, the following have been recovered in good yield from their digitonides, precipated from 90% ethanol: Δ^4- and Δ^5-cholestenone, cholestanone, Δ^4-β-sitostenone and progesterone. Coprostanone and Δ^1-cholestenone gave no precipitate. These digitonides were only precipitated at concentrations much greater than those sufficing for cholesterol. Defining *Digitonin-Precipitability* (*DP*) as $1000/c$, where c is the minimum concentration (mg./100 ml.) at which visible precipitation occurs in 24 hr. at 18–20°, the figures are approximately: cholestanol and cholesterol, >5000; coprosterol, 167; cholestanone and Δ^5-cholestenone, 17; Δ^4-β-sitostenone, 6·2; Δ^4-cholestenone, 2·8 and progesterone, 1·0. These factors may be useful in studies of steroid metabolism.

Butenandt & Mamoli (1935) showed that 3:20-diketo-*allo*pregnane and also 3 (β)-acetoxy-20-keto-*allo*pregnane gave digitonides: however, the more general nature of the reaction was apparently not appreciated.

For steroids with oxygen at C_3, digitonin-precipitability seems to depend on (1) suitable polarity at C_3, (2) the shape of the molecule and (3) the solubility of the steroid.

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DEMONSTRATIONS

Estimation of 'Pregnanediol-like Glucuronide' in Human Urine. By N. G. Bisset, B. W. L. Brooksbank and G. A. D. Haslewood. (*Guy's Hospital Medical School, S.E.*1)

An Inorganic Grey Solution. By L. C. Thomson (introduced by G. A. D. Haslewood). (*Guy's Hospital Medical School, S.E.*1)

This solution is described in *Trans. Faraday Soc.* (1946), p. 663.

A Method of Estimating Peptic Activity in Gastric Contents. By J. N. Hunt (introduced by G. A. D. Haslewood). (*Guy's Hospital Medical School, S.E.*1)

The Electrometric pH Determination of Sound and Carious Dentine. By P. Pincus and J. Jackson. (*Westminster Hospital Medical School, London, S.W.*1)

Few accounts of estimation of pH values of sound or carious dentine in situ, appear in the literature. In the present demonstration, fine holes are drilled in the tooth at the edge of the carious lesion and pH values within these holes determined by quinhydrone, using a fine gold wire. For ease of adjustment, movement of the electrode is controlled by screw motions in three directions. A suitable electrode touches the dentine elsewhere. Possible errors in the quinhydrone electrode measurements are considered.

(A) Some Reactions of Thetine. (B) Evolution of Dimethyl Sulphide from Seaweed. By F. Challenger and M. I. Simpson. (*The University, Leeds*)

The **258th Meeting** *of the Biochemical Society was held at the Rowett Research Institute, Bucksburn, Aberdeen, on* 11 *April* 1947 *at* 2.30 *p.m., when the following papers were read:*

COMMUNICATIONS

Cobalt as a Growth Factor. By James Stewart. (*Animal Diseases Research Association, Moredun Institute, Edinburgh* 9)

It is now established that lambs reared on pasture which has a cobalt content of less than ten parts per hundred million are liable to suffer from a debilitating disease called 'pining' or 'vinquish'. The physiological role of cobalt is still unknown. During 1946 a first attempt was made to induce cobalt-deficiency in lambs by feeding an artificial diet low in cobalt. Ewes were fed for the last 3 months of pregnancy on a diet containing 0·068 mg. cobalt per day, whilst a control group receiving the same diet was dosed by the mouth with 10 mg. cobalt weekly. Three lambs from the control group made normal growth reaching a live weight of over 100 lb. in 40 weeks. They were dosed with 10 mg. cobalt weekly and had access to the artificial diet from the eighth week of life. Only two lambs were born, or survived for a few days after birth, to the experimental group of ewes receiving only the cobalt-deficient diet. Both made normal progress till about the sixteenth week of life when one began to lose weight and died at the twenty-fifth week with all the symptoms of cobalt-deficiency and weighing 38 lb. The other experimental lamb did not lose weight but made no increase in weight from the sixteenth to the thirty-first week. At this stage the three control lambs averaged 73 lb. live weight and the experimental lamb 41 lb. The experimental lamb was given 10 mg. of cobalt weekly from this date and immediately began to increase its live weight, adding 15 lb. in 10 weeks.

It would appear from this experiment that (1) an artificial diet containing 0·068 mg. cobalt per day will produce all the symptoms of cobalt-deficiency in lambs, and (2) cobalt is a factor essential for growth in lambs.

Determination of Glutamine in Quantities of some Millimicro gram-molecules, and a Demonstration that Inhibition of the Synthesis of Glutamine by Streptococci is Associated with Inhibition of their Growth. By H. McIlwain, J. A. Roper and D. E. Hughes. (*M.R.C. Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield* 10)

Glutamine in quantities of a few μmol. in 1–10 ml. can be determined by chemical methods (cf. Archibald, 1945), depending, for example, on the lability of its amide group. For studies of the metabolism of glutamine in micro-organisms (McIlwain, 1946) a more sensitive method of determining the substance was required, and the determination has been accomplished by using bacterial growth in the presence of a differential inhibitor. Strains of virulent haemolytic streptococci require glutamine for growth (McIlwain, Fildes, Gladstone & Knight, 1939), but unless frequently passaged they are capable of using ammonium glutamate in its place. Apart from this, their response to glutamine is highly specific (McIlwain, 1939). α-Amino-γ-methylsulphonylbutyric acid (the sulphoxide derived from methionine) was found by Borek, Miller, Scheiness & Waelsch (1946) and Waelsch, Owades, Miller & Borek (1946) to inhibit the growth of lactobacilli from nutrients which included ammonium glutamate, though growth from glutamine was not affected. A similar relationship has now been shown to obtain in the haemolytic streptococci, and the growth of these organisms in the presence of the sulphoxide has been applied as a method for determining glutamine. Growth from unknown materials and from quantities of glutamine between 5 and 40 mμmol. are measured photometrically after 1–2 days.

By using this method it has been possible to demonstrate synthesis of glutamine by non-proliferating suspensions of streptococci which were capable of growth from nutrients including ammonium glutamate, though no synthesis was found in organisms requiring added glutamine for growth. Also, the action of methionine sulphoxide as an inhibitor of bacterial growth has been shown to be associated with its inhibition of this synthesis. In the case of a strain of *Streptococcus faecalis*, the sulphoxide (0·028M) depressed the synthesis of glutamine from ammonium glutamate (0·0028M) and glucose (0·0167M) to 50% of its uninhibited rate, and from 0·00055M ammonium glutamate and glucose to 28%.

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Sources of Energy for the Bacteria of the Bovine Rumen. By M. L. McNaught and J. A. B. Smith. (*Hannah Dairy Research Institute, Ayr*)

In earlier work, when rumen contents were incubated *in vitro*, the conversion of non-protein N to protein exceeded the breakdown of protein to ammonia only when a suitable source of energy was readily available. In that work starch, dextrin, maltose and glucose were all readily utilized. Several other substances have now been tested, and it has been found that only those compounds which contain an aldehyde or potential aldehyde group are utilized. Hydroxy and carboxylic acid derivatives of the sugars appeared to be of no value as sources of energy for the bacteria, nor were the lower fatty acids which are known to be formed in significant amounts in the rumen. The naturally occurring pentoses *l*-(+)-arabinose and *d*-(+)-xylose were both utilized, but *d*-(−)-arabinose was not.

Influence of the Extraction Rate of Flour on Conditioned Pyridoxine Deficiency in Rats. By M. B. Richards (introduced by W. Godden). (*Rowett Research Institute, Aberdeen*)

A preliminary report on this work (Richards, 1946) showed that the conditioned pyridoxine deficiency caused in rats by excessive intake of vitamin B_1 on a diet containing 61% of white flour (72% extraction) diminished in degree as the extraction rate of the flour was raised from 72 to 77, 80 and 85%.

Further breeding tests with the does reared from weaning on the experimental diets showed that in the second and third litters there was no progressive deterioration but rather a tendency towards improvement indicated by a decrease in the number of observed fits in the suckling rats and an increase in their weaning weights. But second-generation breeding tests revealed very marked degeneration in the group receiving 72 % flour, only sixteen young surviving to weaning from two successive matings of ten does. Considerable degeneration was evident also in the groups receiving 77 and 80 % flour, and in that receiving 72 % flour plus pyridoxine. In these groups 2–4 does out of 10–11 failed to rear litters, even when given a second mating. In the group receiving 85 % flour there were no failures and there was little evidence of degeneration except for a slight decrease in the weaning weights of the young. Each of the twelve does reared a litter, with an average of 7·4 rats per litter. In this breeding test the performance of the 77 % group was slightly better than that of the 80 % group, but in a third-generation test the 77 % group showed almost complete failure, only two litters being reared from eleven does, while in the 80 % group six litters were reared from nine does. In the group fed 72 % flour with a supplement of pyridoxine six litters were reared from eleven does. It seems probable that the degeneration evident in this group is due to deficiencies of B-factors other than pyridoxine. The group on 85 % flour maintained its good performance: again none of the does failed to rear a litter. Thirteen litters averaging 7·6 rats per litter with an average weaning weight of 41·5 g. were reared—all without fits.

These experiments furnished additional evidence of the reduction in weight of the thymus of weanling rats previously reported in conditioned pyridoxine deficiency and of the beneficial effect of transferring rats from 72 to 85 % flour or supplementing the diet of 72 % flour with pyridoxine. To what extent this effect is specific for pyridoxine has not yet been determined, but experiments are at present in progress in which individual B-factors are being given as supplements to the diet with high B_1 and 72 % flour. So far, the results indicate that pyridoxine has a significantly greater effect in promoting the growth of the young rats than any of the other supplements administered.

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Some Factors Affecting Chemical Detection of Hydrogen Peroxide Formed by Oxidative Deamination of Certain Amino-acids by Spermatozoa. By J. Tosic. (*School of Agriculture, University of Cambridge*)

It has been shown recently that bull spermatozoa oxidatively deaminate *l*-phenylalanine, *l*-tyrosine and *l*-tryptophan with simultaneous formation of H_2O_2 and NH_3 (Tosic, 1947). The H_2O_2 has an inhibitory effect upon motility and respiration of the spermatozoa. If the concentration of accumulated H_2O_2 is as low as 10^{-2} μmol. H_2O_2/1 ml. supernatant, it can still be detected chemically by a positive benzidine-peroxidase reaction (Tosic & Walton, 1946). It was noticed, however, that if a glycolysable carbohydrate were also present with substrates which can undergo oxidative deamination, the benzidine-peroxidase reaction was negative, although the formation of H_2O_2 could be detected by its effect upon motility and aerobic metabolism of spermatozoa; lactic acid, which is formed by spermatozoa from glucose, fructose and mannose (Redenz, 1933; Mann, 1946) produced a comparable effect. This negative result of H_2O_2 detection was not due to 'interference' with the reaction by the sugars or lactic acid, since the presence of low concentrations of H_2O_2 could still be detected, and higher concentrations quantitatively recovered, after incubation with glucose, fructose or lactate in the absence of living spermatozoa. Acetate, propionate, succinate, fumarate, malate and citrate did not produce this effect either in presence or absence of living sperm suspensions.

Bovine spermatozoa can oxidize lactic acid, and in this oxidation pyruvic acid seems to be an intermediate, which is further oxidized by the sperm at a rate somewhat higher than that of lactate oxidation. However, pyruvic acid and H_2O_2 are known to react (Fenton & Jones, 1900) according to equation given by Holleman (1904) and Negelein & Brömel (1939):

$$CH_3.CO.COOH + H_2O_2 = CH_3.COOH + CO_2 + H_2O. \quad (I)$$

The rate of this reaction, which requires no catalyst, is higher than the rate of oxidation of comparable concentrations of pyruvate by spermatozoa. Pyruvic acid formed, therefore, can enter into reaction with H_2O_2 present, and thus eliminate it from the system. If pyruvic acid formation from lactic acid is inhibited in spermatozoa by different concentrations of sodium barbiturate, which is known to inhibit lactic acid dehydrogenase in brain cortex

(Quastel & Wheatley, 1932), then there is a correspondingly sparing effect in H_2O_2 elimination from the system.

The conclusion is, therefore, that in metabolic reactions in which formation of H_2O_2 and pyruvic acid takes place, the accumulation of H_2O_2 and its chemical detection will depend, among other factors, also on the ratio of the rates of H_2O_2 and pyruvate formation to the rates of their elimination as in equation (I) and other possible reactions.

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Conversion of β-Carotene into Vitamin A in the Intestine of the Rat. By J. Glover, T. W. Goodwin and R. A. Morton. (*Department of Biochemistry, University of Liverpool*)

The site of conversion of β-carotene to vitamin A in the animal has generally been assumed to be the liver (e.g. Moore, 1931; Bessey & Wolbach, 1938), although *in vivo* and *in vitro* experiments have given negative or equivocal results (e.g. Olcott & McCann, 1931; Rea & Drummond, 1932; Drummond & MacWalter, 1933; Parienti & Ralli, 1932).

A recent observation (Ball, Glover, Goodwin & Morton, 1946) that retinene$_1$ (vitamin A aldehyde), which is a possible intermediate, is rapidly converted to vitamin A in the intestinal wall of the rat prompted a test of the alternative hypothesis that the provitamin → vitamin transformation occurs in the gut. The work of Sexton, Mehl & Deuel (1946), who administered β-carotene to depleted animals by routes other than oral, demonstrated the appearance of carotene in the liver without alleviation of avitaminosis A. They suggest that this negative evidence excludes the liver and points to the gut as the site of transformation. Direct evidence that the process occurs in the gut wall is now submitted.

Large doses (5–15 mg.) of crystalline β-carotene in arachis oil were administered orally to rats which, judged by stationary weight and absence of vitamin A from the livers of control animals, were avitaminotic. The animals were killed at 5–24 hr. after dosing and the intestines and livers removed. The small intestines, freed from lumen contents, were saponified and the unsaponifiable matter chromatographed to remove carotene. The resulting material was cooled to −80° to remove sterols and then tested for vitamin A.

Preliminary tests showed that positive results were obtained on animals killed 6–8 hr. after dosing. Four further animals received 5 mg. each of β-carotene and the *pooled* intestinal unsaponifiable fractions contained 25 i.u. of vitamin A judged both by the 617 mμ. band ($SbCl_3$ colour test) and the selective absorption at 328 mμ.

It was found that some (100 i.u.) of vitamin A had already reached the livers. The small intestines of animals killed 6 hr. after oral administration of 14 mg. β-carotene in oil were therefore incubated intact at 37° and then treated as above and more than 20 i.u. of vitamin A were found in *each* intestine.

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Iodine Metabolism in Relation to Dietary Calcium. By B. W. Simpson (introduced by W. Godden). (*Rowett Research Institute, Aberdeen*)

A pilot iodine balance experiment on individual adult rats indicated that an increase of urinary iodine resulted from the inclusion in the diet of Ca (as $CaCO_3$) in excess of the adult requirement.

The present experiment was designed to determine whether, over a prolonged period of time, the excretion of iodine by adult rats and the iodine content of their thyroid glands were significantly

affected by the level of Ca (as $CaCO_3$) in the diet.

Six adult female stock rats were divided into three pairs and fed a low-calcium, low-iodine diet, to which was added $CaCO_3$ at three levels, to provide total Ca contents of 0·05, 0·50 and 1·50 g./100 g. food. The rats were 10 months on these diets and each had two litters in that time, after which balances involving the estimation of iodine in the diet, urine and faeces, were carried out weekly on each pair over a period of 6 weeks. The following table presents the cumulative iodine balances for 6 weeks, the balance study following a pre-period of 1 week in the metabolism cages for adjustment.

The excretion of iodine by adult rats was increased, and the iodine content and concentrations in the thyroids were diminished, by feeding a moderately high or high percentage of Ca (mainly as $CaCO_3$) in the diet.

Cumulative iodine balances on three levels of calcium intake

Diet	Iodine intake (γ)	Iodine output (γ)	Iodine balance (γ)
Low Ca	50·4	28·8	+21·6
Moderately high Ca	48·6	33·6	+15·0
High Ca	47·3	35·4	+11·9

Weight and iodine content of the thyroid glands

Diet		Wt. of thyroid (g.)	Wt. of thyroid/ 100 g. body wt. (g.)	Iodine content (γ)	% I_2
Low Ca	(1)	0·036	0·014	3·47	0·010
	(2)	0·057	0·023	4·83	0·008
Moderately high Ca	(1)	0·084	0·031	1·96	0·002
	(2)	0·050	0·019	1·42	0·003
High Ca	(1)	0·084	0·030	1·48	0·002
	(2)	0·056	0·021	2·16	0·004

DEMONSTRATIONS

Conditioned Pyridoxine Deficiency in Rats. By M. B. Richards (introduced by W. Godden)

Bacterial Decomposition of Cellulose in the Rumen. By F. Baker and S. T. Harriss (introduced by D. P. Cuthbertson). (*Rowett Research Institute, Aberdeen*)

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **259th Meeting** *of the Biochemical Society was held in the Medical School, Hospitals Centre, Birmingham* 15, *on Saturday,* 7 *June* 1947, *when the following papers were read:*

COMMUNICATIONS

Shrinkage of Collagen. By F. G. Lennox. (*Biochemistry Section, Division of Industrial Chemistry, Council for Scientific and Industrial Research, Melbourne, Australia*)

Studies of the shrinkage of collagen in sheepskin, skin 'collagen' preparations and rat-tail tendon have shown that the temperature at which shrinkage commences (the shrinkage temperature or s.t.) increases with the load applied per unit cross-sectional area and is higher at a given stress for skin than for tendon fibres. It is elevated by removal of water but is unaffected by variation in pH value of the external solution between 4·5 and 10·5. Outside these limits the s.t. falls sharply. Attempts to remove non-collagenous constituents of skin by treatments involving alkali extraction lowered the s.t. The majority of inorganic ions tested at 1·0M concentration and pH 7·0 lowered the s.t. Their relative effectiveness agreed in general with other lyotropic effects, the lowest s.t. being produced by CNS^- and the highest by $SO_4^=$. At pH values below 2·5, however, the relative effectiveness on skin of anions which lowered the s.t. at neutrality was reversed, CNS^- producing the highest s.t. and Cl^- the lowest. Reversion with change of pH value was not observed with cations nor in experiments with tendon.

Under the high stress at which tendon fibres were tested, many of them ruptured in acid solution at a temperature coinciding with the s.t. of other fibres tested under the same conditions. Rupture was most pronounced at an external pH of 2·5–4·5, and is suspected of being due to the weakening of some interfibrillary binding substance.

After shrinking in 10M-KCNS at 18° the tissues displayed marked elasticity, but after shrinking in water at 70° and cooling only the skin collagen preparation could be stretched as readily as when immersed in the KCNS solution.

Of the organic compounds tested at 1·0M concentration and pH 7·0, only those which are well ionized lowered the s.t., and, as with the inorganic salts, anionic compounds were the more effective. Except for formic, acetic and propionic acids, all the fatty acids and aliphatic amines tested lowered the s.t., its value being reduced progressively with increase in length of the C chain. All the aliphatic dicarboxylic acids examined elevated the s.t., and amongst the aromatic acids s.t. lowering was reduced by the introduction of a second ionized acid group into the structure. Conversely, s.t. lowering by aliphatic or aromatic cations was unaffected by the introduction of additional ionized basic groups into the molecule. The lowest s.t.'s were obtained with aromatic anions having a well-ionized 'head' and an unsaturated group in the side chain carrying the head, as in phenylpropiolic acid and cinnamic acid, or an unionized hydrophilic group attached to the ring and preferably in the *ortho* position, as in salicyclic acid and anthranilic acid.

Reduction of the s.t. is attributed to competitive adsorption of the ions, liberated from the added salt, on the oppositely charged components of the salt links in collagen, between the arginine and lysine residues on the one hand and the glutamic acid and aspartic acid residues on the other.

The Effect of Calciferol on Serum Calcium and its Relation to Abnormal Calcification. By J. Dawson and Doris E. Dolby. (*Department of Biochemistry, School of Medicine, Leeds* 2)

The effect of activated sterols on the calcium level of blood has been previously investigated. A review by McLean (1941) described toxicity effects and a rise in total serum calcium which our results confirm. Calciferol treatment of lupus vulgaris provided material for an investigation of any changes in serum calcium. As well as total serum calcium, the diffusible calcium was estimated in each case by the method of Updegraff, Greenberg & Clark (1926). In some cases the ionic calcium was also determined by the frog-heart technique. A general rise in total serum calcium was observed, but this level could not be correlated with dosage of vitamin D, nor with the appearance of toxic symptoms. Figures for diffusible calcium revealed that whenever symptoms occurred there was always a marked rise in the values obtained. In those cases where ionic calcium levels were recorded these were within normal limits.

Vitamin D therapy tended to raise the serum-protein level. In certain cases the serum protein and total serum-calcium levels were both raised, but the diffusible calcium remained within normal limits. This rise in serum protein apparently increases the amount of bound calcium and thus diminishes the rise in diffusible calcium level.

The precipitate obtained by adding oxalate to the diffusate of serum was investigated. After ether extraction the material was hydrolyzed by 2N-sulphuric acid. In cases where the diffusible calcium level was raised, the resulting solution was found to give a positive Liebermann-Burchard reaction. A repetition of this experimental procedure on serum from hypervitaminotic D rabbits yielded material which was ether extractable and gave a positive Liebermann-Burchard reaction. Thus it would appear possible that in cases of hyper-vitaminosis D, part of the diffusible calcium fraction is found in association with a steroid component.

Cases of abnormal calcification were then examined. Cases of Paget's disease, recently diagnosed, showed a raised diffusible calcium level; in older-standing cases, where symptoms were absent and the disease believed to be quiescent, this rise was not observed. Similarly, the diffusible calcium level was raised in a case of massive arterial and arteriolar calcification. This would seem to support the view that some fraction of the diffusible calcium is intimately concerned in calcification, and as the commonest described toxic manifestation of calciferol is metastatic calcification, it may well be the component associated with steroid, which is of importance.

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The Nature of the Tryptophanase Complex. By E. A. Dawes, J. Dawson and F. C. Happold. (*Department of Biochemistry, School of Medicine, Leeds* 2)

The preparation of a cell-free extract of tryptophanase and its resolution into coenzyme and apoenzyme has been effected (Dawes, Dawson & Happold, 1947). The study of the effect of various inhibitors on tryptophanase activity initially provided strong presumptive evidence for the nature of the enzyme complex. Mepacrine inhibition and its antagonism by riboflavin (Dawson, 1946) gave indication of the functioning of riboflavin as a coenzyme factor, while the effect of characteristic carbonyl reagents pointed to the presence of an aldehyde- or carbonyl-containing compound, and this has been reconciled with the presence of pyridoxal phosphate, as revealed by spectrophotometric analysis. In a similar way inhibition by benzamide, reversed by nicotinic acid, could be related to the presence of a pyridino component. *Esch. coli* contains no catecholase and is incapable of oxidizing dimethyl-*p*-phenylenediamine (Happold, 1930). Inhibition produced by cyanide, carbon monoxide and hydrogen sulphide suggested that a cytochrome type of system is operating, though it is known from the work of Keilin & Harpley (1941) that cytochrome *c* and cytochrome oxidase are absent from *Esch. coli* and also that bands *b* and *c* of normal cytochrome are replaced by one band which lies at 560 mμ and which represents the α band of non-autoxidizable haemochromogen component b_1.

Verdoperoxidase (VPO), prepared by the method of Agner (1943), has been shown to have a stimulatory effect upon indole production from tryptophan by our cell-free extract. It has been suggested by Keilin (1933) and Kuhn (1934) that VPO might be considered as more or less modified cytochrome *a* derivatives, and hence this finding is of considerable interest. The inhibitory effect of British Anti-Lewisite (2:3-dimercaptopropanol) and sodium azide is compatible with the presence of an iron-containing system such as VPO. Spectrochemical investigation of an unpurified sample of apoenzyme revealed the presence of iron, but not copper.

Riboflavin, pyridoxal phosphate and diphosphopyridine nucleotide (DPN) are each capable of reactivating the apoenzyme; stronger activation is obtained with pyridoxal phosphate plus DPN and riboflavin plus DPN, whereas the maximum effect is obtained by the addition of all three.

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The Metabolism of Synthetic Oestrogens (Stilboestrol, Dienoestrol and Hexoestrol) in the Rabbit. By K. S. Dodgson, G. A. Garton and R. T. Williams. (*Department of Biochemistry, University of Liverpool*)

Stilboestrol. In 1942 Mazur & Shorr described the isolation of stilboestrol monoglucuronide from the urine of rabbits injected with small doses of stilboestrol for a fortnight. These workers dried the compound at 110° before analysis and therefore described the compound as being anhydrous, m.p. 175°. We have now isolated the glucuronide in excellent yields (1 g. Na salt/g. stilboestrol) by ether extraction from rabbit urine after single oral doses (0·8 g.). About two-thirds of the excreted glucuronide occurs in the urinary deposit as the sparingly soluble sodium salt. The crystalline glucuronide (needles) is a *dihydrate* ($C_{24}H_{28}O_2$, $2H_2O$) sintering at 70–100°, m.p. 179° and $[\alpha]_D^{20°} - 56·5°$ (in ethanol). Quantitative estimations showed that about 70 % of stilboestrol (150 mg./kg.) is excreted as a glucuronide, this being excreted up to 4 days after dosing, the maximum excretion occurring on the second day after dosing. No detectable change was found in the ethereal sulphate and neutral sulphur output.

Hexoestrol. Hexoestrol is also excreted as a glucuronide, but the isolation of the latter from urine cannot be achieved in the same way as for the stilboestrol derivative, because it is insoluble in ether. As a model for isolating hexoestrol glucuronide, a study was first made of the glucuronide of *p*-hydroxydiphenyl. *p-Glucuronosidodiphenyl* (*p*-phenylphenyl-glucuronide, m.p. 189°) was isolated by extracting acidified urine with 1:3 ethanol/ether mixtures. *Hexoestrol monoglucuronide* (m.p. 179–180°) was isolated in a similar manner. Quantitative results showed that about 66 % hexoestrol was excreted as a glucuronide. No change in the ethereal sulphate and neutral S output was detected.

Dienoestrol. Quantitative determinations with orally administered dienoestrol (m.p. 238–239°) indicated no detectable change in the glucuronic acid, ethereal sulphate or neutral S output in rabbits. This may not mean that a glucuronide is not formed, but that the rate of absorption of dienoestrol is so slow that the normal output of glucuronic acid is hardly disturbed. Much orally administered dienoestrol appears in the faeces.

Although quantitative determinations of glucuronic acid output, within the limits of the method used, indicate that dienoestrol behaves differently from hexoestrol and stilboestrol, we are not yet prepared to say that dienoestrol does not form a glucuronide. Not one of the three appears to form an ethereal sulphate, but here again a slow rate of absorption may make alterations in the ethereal output insignificant. Mercapturic acids are unlikely to be formed for all three compounds are *para*-substituted.

Degradation to *p*-hydroxybenzoic acid is a possible fate of these compounds, but this acid has not been detected in the urine and our results indicate that such a degradation probably does not take place.

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The Oxidation Products of Orthanilamide in the Rabbit. By A. L. Stubbs and R. T. Williams. (*Department of Biochemistry, University of Liverpool*)

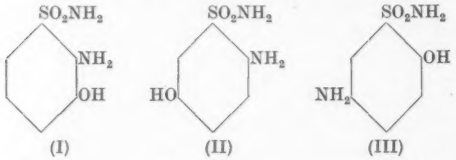
Williams (1945) reported that the oxidation product of orthanilamide (*o*-aminobenzenesulphonamide) in the rabbit was probably 3-hydroxy-2-amino-benzenesulphonamide. It has now been found that there are two oxidation products, both of which are excreted conjugated.

(1) *Hydroxyorthanilamide*-A (m.p. 188°) was isolated from a fraction of orthanilamide urine which contained mainly conjugated sulphates. 'Orthanilamide urine' is very rich in ethereal sulphates, and in four experiments the following figures were obtained for the percentage of the dose of orthanilamide excreted as ethereal sulphates: (1) 46 %, (2) 44 %, (3) 30 % and (4) 39 %.

Hydroxyorthanilamide-A has also been synthesized by the following route: metanilamide → *m-hydroxybenzenesulphonamide* (m.p. 166°) → *nitro-m-hydroxybenzenesulphonamide* (m.p. 175°) → *hydroxyorthanilamide*-A (m.p. and mixed m.p. 188°). Hydroxyorthanilamide-A could be therefore 3-hydroxy- or 5-hydroxyorthanilamide. Spectroscopic and other considerations indicate that it is probably the 3-hydroxy derivative (1).

(2) *Hydroxyorthanilamide*-B (m.p. 179–180°) was isolated by chromatography from an ether extract of acid hydrolyzed orthanilamide urine (glucuronide and ethereal sulphate fractions). It is believed to be 5-hydroxyorthanilamide (5-hydroxy-2-amino-ben-

zenesulphonamide (II)) for its absorption spectrum in alcohol is almost identical with that of 2-hydroxy-5-amino-benzenesulphonamide (III) (Williams, 1942).



Ultra-violet absorption spectra of the above compounds in ethanol:

(I) (*a*) biological sample: $\lambda_{max.}$ 310 mμ (log $\epsilon_{max.}$ 3·66) and ~235 mμ (log $\epsilon_{max.}$ 3·8),

(*b*) synthetic sample: $\lambda_{max.}$ 312 mμ (log $\epsilon_{max.}$ 3·64) and ~235 mμ (log $\epsilon_{max.}$ 3·77).

(II) $\lambda_{max.}$ 245 mμ (log $\epsilon_{max.}$ 3·845) and 326 mμ (log $\epsilon_{max.}$ 3·54).

(III) $\lambda_{max.}$ 243 mμ (log $\epsilon_{max.}$ 3·84) and 325 mμ (log $\epsilon_{max.}$ 3·47).

(3) *Other excretion products.* Free orthanilamide (m.p. 156° after recryst.) can be extracted from the urine with ether. The amount excreted is uncertain, but material yielding orthanilamide on repeated crystallization can be obtained by ether extraction weighing up to 40 % of the dose. We were unable to ascertain whether orthanilamide is acetylated *in vivo*. No acetylorthanilamide could be isolated and determinations of 'free' diazotizable amino groups and 'total' diazotizable amino groups after acid hydrolysis yielded no information on this point. In six experiments, at a dose level of 0·1 g./kg., the 'free' amino groups were equivalent to 73 % of the dose, whereas the 'total' amino groups were 66 %. The slight drop in the figure for total amino groups is no doubt due to the conjugated hydroxy orthanilamides giving a more intense colour than the free compounds.

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The Conjugation of *d*-Adrenaline and certain Catechol Derivatives in the Rabbit. By K. S. Dodgson, G. A. Garton and R. T. Williams. (*Department of Biochemistry, University of Liverpool*)

It has been suggested by Richter (1940, 1941–2) that adrenaline is inactivated in the body by conjugation rather than by oxidative deamination. Richter's work also suggests that *d*- and *l*-adrenaline are excreted by man as ethereal sulphates. Furthermore, Deichmann (1943) claims that *l*-adrenaline is excreted as a sulphate in rabbits. However, an examination of the data for these claims shows that whilst conjugation does take place, the evidence is not unequivocally in favour of a sulphate conjugation.

We have determined the glucuronic acid and ethereal sulphate in rabbits after large oral doses of *d*-adrenaline (m.p. 210°; $[\alpha]_D^{20°}$ + 50° in N/10-HCl). The results are given in the following table:

Rabbit no.	Dose (mg./kg.)	% dose *d*-adrenaline excreted as: Ethereal sulphate	Glucuronide
101	250	0	—
68	250	4·3	18·9
102	250	6·7	19·7
60	200	0	19·1
67	200	6·9	29·6
68	200	—	20·5

This table shows that whereas about 21 % of *d*-adrenaline is excreted as a glucuronide, the amount excreted as a sulphate is insignificant.

The glucuronide has been isolated as a crude barium salt, but we are not yet in a position to discuss its nature, except to say that on hydrolysis it yields material giving colour tests for adrenaline or some closely related compound. No unchanged *d*-adrenaline was excreted and the conjugated material was excreted within 24 hr. of dosing.

The figures 4·3, 6·7 and 6·9 % for the sulphate conjugation in three cases are probably not significant for the error of the method is ± 5 %.

Although *d*-adrenaline does not appear to conjugate with sulphate, catechol and protocatechuic acid do so (see following table):

Rabbit no.	Compound	Dose (mg./kg.)	% of dose excreted as a monosulphate
97	Catechol	100	18·9
98	Catechol	100	16·5
80	Protocatechuic acid	230	15·6
18	Protocatechuic acid	250	13·9

These two compounds also form glucuronides and 20 % of protocatechuic acid can be recovered from urine unchanged in the crystalline state.

Whether *l*-adrenaline forms a glucuronide like *d*-adrenaline has yet to be investigated, but one has to bear in mind the finding of Lipschitz & Bueding (1939) that *l*-adrenaline suppresses glucuronic acid conjugation in liver slices.

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The Fate of *p*-Hydroxybenzylamine and some Related Compounds in the Rabbit. By R. L. HARTLES and R. T. WILLIAMS. (*Department of Biochemistry, University of Liverpool*)

Hartles & Williams (1946, 1947) have shown that the substituted benzylamines, Ambamide (Marfanil) and V 335 are inactivated *in vivo* by deamination and oxidation to the corresponding acids. The present work was designed in order to detect the intermediate aldehyde stage in the deamination and oxidation of an amine. Earlier work had suggested (cf. vanillin, Sammons & Williams, 1941) that *p*-hydroxy aromatic aldehydes were slowly oxidized *in vivo*, and in the case of vanillin, a conjugated aldehyde was excreted. Hence a study of *p*-hydroxybenzylamine might enable us to detect the intermediate aldehyde in the urine.

When *p*-hydroxybenzaldehyde is fed to rabbits, its glucuronide is excreted in the urine in small amounts (isolated as a 2:4-*dinitrophenylhydrazone*, m.p. 165°). On feeding *p*-hydroxybenzylamine, the hydroxy aldehyde can be detected in the urine by the colour reaction of Sammons & Williams (1941), but so far we have failed to isolate it.

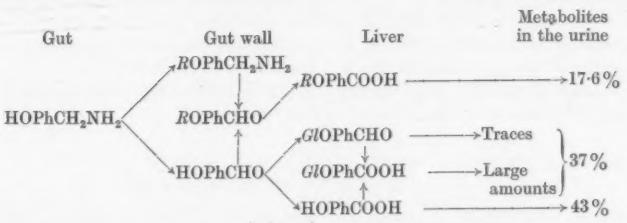
Another approach to the problem was made by studying the fate of *p*-hydroxybenzylacetamide and the metabolites of *p*-hydroxybenzylamine. The results obtained are given in the next column.

p-Hydroxybenzylacetamide is not deacetylated and *p*-hydroxybenzylamine is not acetylated by the rabbit. The glucuronide of the former has been characterized as the *methyl ester* of 2:3:4-*triacetyl-p-acetaminomethylphenylglucuronide* (m.p. 174°).

The glucuronic acid conjugations of *p*-hydroxybenzylamine and *p*-hydroxybenzaldehyde are the same, but the sulphate conjugation of the amine is twice that of the aldehyde (cf. Williams, 1938). Sulphate conjugation is known to occur in the intestine as well as in the liver, whereas glucuronide formation occurs only in the liver. The figures in the table below may be interpreted as meaning that (1) *p*-hydroxybenzylamine is partly sulphated in the gut wall before it is deaminized, (2) after deamination it is converted to *p*-hydroxybenzaldehyde which is partly conjugated with glucuronic acid in the liver before oxidation to *p*-hydroxybenzoic acid and (3) if *p*-hydroxybenzylamine were not deaminated before reaching the liver, its glucuronide conjugation could be expected to be similar to *p*-hydroxybenzylacetamide.

Compound	% of dose excreted as Glucuronide	% of dose excreted as Ethereal sulphate
(1) HO-C$_6$H$_4$-$CH_2NHCOCH_3$	72	12·0
(2) HO-C$_6$H$_4$-CH_2NH_2	37	17·6
(3) HO-C$_6$H$_4$-CHO	33	9·2
(4) HO-C$_6$H$_4$-COOH	18	9·5

Schematically the fate of *p*-hydroxybenzylamine could be represented tentatively as follows:



where $R = -SO_3H$ and $Gl = -C_6H_9O_6$.

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Some Observations on Salicylate Therapy in Children. By G. F. MAGGIONI (introduced by E. M. HICKMANS). (*The Children's Hospital (Biochemical Department), Birmingham*)

The argument about the value of salicylate therapy in acute rheumatism (Hanzlik, 1926) has been resumed following Coburn's (1943) recent statement that high doses of sodium salicylate (0·19–0·14 g./kg. body weight) sufficient to raise the blood level to a 'critical' value of 35–45 mg./100 ml., modified the 'sterile inflammatory reaction which occurs during activity of rheumatism' and thus prevented the development of cardiac disease.

Considering the frequency of rheumatic fever in childhood, the problems concerned with salicylate administration are of particular interest. In this paper some observations are presented on the use of these drugs in normal and rheumatic children without any attempt to discuss the efficiency and the specificity of this therapy. The methods used for the estimation of 'salicyl' were, with slight modifications, those of Brodie, Udenfriend & Coburn (1944) for blood and those of Lester, Lolli & Greenberg (1946) for urine.

(1) Sodium salicylate mixtures in water deteriorate on keeping, even on ice. Diminution of 13 % in one month was observed.

(2) Control tests of (*a*) solutions kept in wards (not on ice), and (*b*) method of administration (shaking insufficiently and residue in glass) show a loss of salicylate amounting to 25 %.

(3) Absorption rate of sodium salicylate in water is rapid in children. After 15 min. it can be detected in blood and in urine. Calcium aspirin is absorbed by children, as in adults, more slowly.

(4) The time required for complete excretion of a single dose of sodium salicyl (1 g.) is 30–36 hr. in a normal child. The danger of any cumulative effect and the occurrence of toxic symptoms is therefore small: on stopping administration after many weeks' treatment (10), the drug completely disappears from the blood and urine in 48–72 hr.

(5) The quantity of 'Salicyl' recovered in urine after a single dose is in normal controls about 75 % of intake. In children suffering from acute rheumatism the quantity recovered daily is about 50–60 % or less of the amount taken.

(6) Absorption of sodium salicylate without sodium bicarbonate given per rectum is more than 50 % of intake.

(7) The quantity of sodium salicylate which must be given to children to attain a blood level of 25–35 mg./100 ml. is from 0·12 to 0·18 g./kg. body weight. This dose given continuously (4-hourly, orally) shows individual daily variations in blood level up to 10–12 mg. %.

(8) Sodium salicylate + sodium bicarbonate (1:2) taken by mouth produces within 15 min. the same level in blood as without bicarbonate but the excretion of salicylate in the urine is increased; therefore the blood level decreases more rapidly than without sodium bicarbonate. After 4 hr. the level in the blood is sometimes half of that attained without sodium bicarbonate.

(9) The quantity of sodium salicylate which can be recovered from the urine after giving sodium bicarbonate by mouth in ratio 1:2 is about 50 % more than without sodium bicarbonate in the first 12–18 hr.

(10) There seems to be a relationship between the proportions of the sodium bicarbonate given with the salicylate and the rapidity of output. The greater the proportion of sodium bicarbonate, the quicker the excretion.

(11) Vomiting after administration of salicylate is not necessarily related to the salicylate blood level.

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Hydrolysis of Amides by Liver Extracts. By H. G. BRAY, SYBIL P. JAMES, BRENDA E. RYMAN and W. V. THORPE. (*Department of Physiology, University of Birmingham*)

Previous work in this laboratory has shown that amides differ considerably in their stability to hydrolysis in the rabbit. The relative degree of hydrolysis of various amides by liver extracts under standard conditions has now been studied. This was determined by estimating the acid formed, by a formol titration method, and, in some instances, the ammonia formed, by an aeration method. The results given by the two methods were in good agreement. The following are figures for the relative percentage hydrolysis of amide after 10 hr. under the standard conditions: *p*-nitrobenzamide* 83 %; *m*-

nitrobenzamide* 30%; *o*-nitrobenzamide 28%; benzamide* 27%; asparagine 24%; glutamine 16%; propionamide 15%; nicotinamide 12%; phenylacetamide 8%; *o*-bromobenzamide 7%; *p*-aminobenzamide* 5%; *p*-hydroxybenzamide*, *m*-hydroxybenzamide and *o*-aminobenzamide* 1%; *o*-hydroxybenzamide*, *m*-aminobenzamide*, formamide and acetamide 0%.

In general, these results confirm those obtained in *in vivo* experiments with rabbits in the case of the amides marked with an asterisk. Phenylacetamide is, so far, the only exception.

Metabolism of Aminobenzoic Acids and their Amides. By H. G. Bray, H. J. Lake, F. C. Neale, W. V. Thorpe and P. B. Wood. (*Department of Physiology, University of Birmingham*)

Unlike benzoic acid and its amide (Bray, Neale & Thorpe, 1946), *o*-, *m*- and *p*-aminobenzoic acids and their amides are not metabolically interchangeable in the rabbit. Whereas in benzamide the carbamyl group is readily hydrolyzed and no oxidation products have been detected, in the aminobenzamides the carbamyl group is more stable and oxidation products have been isolated. The position of the amino group influences the quantitative result. Whereas the aminobenzoic acids are not oxidized and conjugated with sulphate, the amides are, 35% of *o*-, 7% of *m*- and 2% of *p*-aminoamide being excreted in this form. The percentage acetylated is much greater with the amides than the acids. Small values for the excretion of ester glucuronide and ether-soluble acid suggest that the carbamyl group in the aminobenzamides is comparatively stable.

N-Acetyl aminobenzamides have been isolated from rabbit urine after administration of *m*- and *p*-aminobenzamides and ester type glucuronides after the administration of *m*- and *p*-aminobenzoic acids. *p*-Aminobenzoyl glucuronide forms a crystalline complex with 1 mol. *p*-aminobenzoic acid.

The oxidation product excreted in urine after giving *o*-aminobenzamide appears to be 2-amino-5-hydroxybenzamide (isolated as 2-amino-5-hydroxybenzoic acid). This lends further support to our hypothesis that oxidation *in vivo* of a disubstituted aromatic compound (in which one substituent is $—NH_2$ or —OH) leads to the formation of a phenol substituted in positions 1, 3 and 4 (Bray, Ryman & Thorpe, 1947). Attempts are being made to isolate the oxidation product of the *m*-amide.

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Metabolism of Vitamin A in Diseases of the Kidneys. By R. Johns, H. Hoch and J. R. Marrack. (*Department of Chemical Pathology, London Hospital, E.* 1)

In a small series of cases we have found that the amount of vitamin A in the liver was low in those with a long history of renal failure and about normal in those with a short history. We have found that patients with severe renal failure may excrete 1000 or more units of vitamin A per day in the urine.

There was no definite increase in the rate of excretion when the concentration in the serum was raised by a large dose of vitamin A.

The resting concentration in the serum of some cases was abnormally high and the increase over normal was due to increase in the amount of free alcohol. After a large dose of vitamin A the rise was confined to the ester fraction.

The vitamin A in the urine was in the form of ester and alcohol; the ratio of ester to total vitamin A was higher in the urine than in the serum taken at the same time.

We suggest that the low levels found in the liver are due to a prolonged drain on the stores due to loss in the urine.

Schmidt-Nielsen Technique for Micro-estimation of Fat. By H. G. Sammons and J. B. Finean

The Mechanism of Fat Absorption in the Rat. By A. C. Frazer, J. M. French and P. E. Sagrott

Distillation of Cyanic Acid as Evidence for the Presence of Cyanate in Red Cells. By P. DIRNHUBER and F. SCHÜTZ. (*Department of Pharmacology, The Medical School, Hospitals Centre, Birmingham* 15)

In the course of an investigation into the possible formation *in vivo* of ammonium cyanate by isomeric transformation from urea ($(NH_4)_2CO \rightarrow NH_4CNO$), and on the actions of cyanate (Schütz, 1945, 1946; Birch & Schütz, 1946), a method was sought for by which small amounts of cyanate could eventually be shown in biological material. It is known that cyanic acid, when set free from cyanate by acidification in aqueous solutions, is at once decomposed into CO_2 and NH_4, a small part being polymerized into cyammelide (see Werner, 1923). We found, however, a way to set cyanic acid free and partly to recover it after distillation. The solution is distilled in vacuum in the usual way at room temperature, the condenser, preferably with double surface, is loosely packed with filter paper strips (Whatman no. 541) soaked in N-NaOH, and cooled with ethanol and solid CO_2. When the vacuum is $< 0{\cdot}05$ mm. Hg, 2M-citrate buffer (pH 5·3) is let in, and, thereafter, the temperature of the water-bath, containing the distillation flask, is raised to 50°. Some of the cyanic acid is then caught on the NaOH papers and remains stable as cyanate.

The presence of cyanate was then shown by converting it into urea. CO_2 was bubbled through the distillate containing the NaOH papers, and an excess of $(NH_4)_2SO_4$ was added. After evaporation to dryness on the water-bath, the residue was finely powdered and extracted with boiling ethanol. This extract was dried down and the residue taken up in H_2O; its urea content was determined manometrically (Krebs & Henseleit, 1932).

When pure solutions of NaCNO were treated as described above, 30 % of the theoretical amount of urea was found. A 10 % yield was obtained when NaCNO was added to lysed red cells. Traces of cyanate were found in normal red cells, but larger amounts could be shown in erythrocytes of nephrectomized rabbits, especially when the water and urea retention became very marked. In these cases an equivalent up to 0·5 % of the urea present was recovered as cyanate in the distillate. Since in pilot runs the yield of added cyanate was usually of the order of 10 %, the real figure is probably much larger. No cyanate was detected in serum. The results provide direct evidence for the presence of cyanate in red cells.

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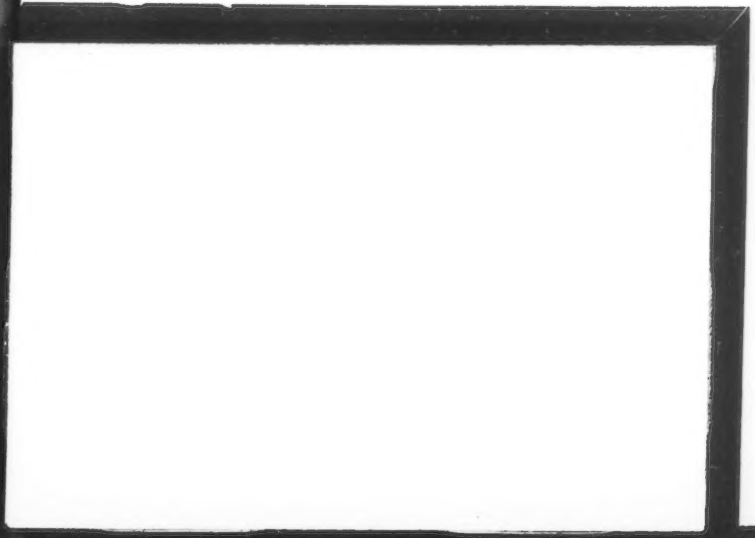
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